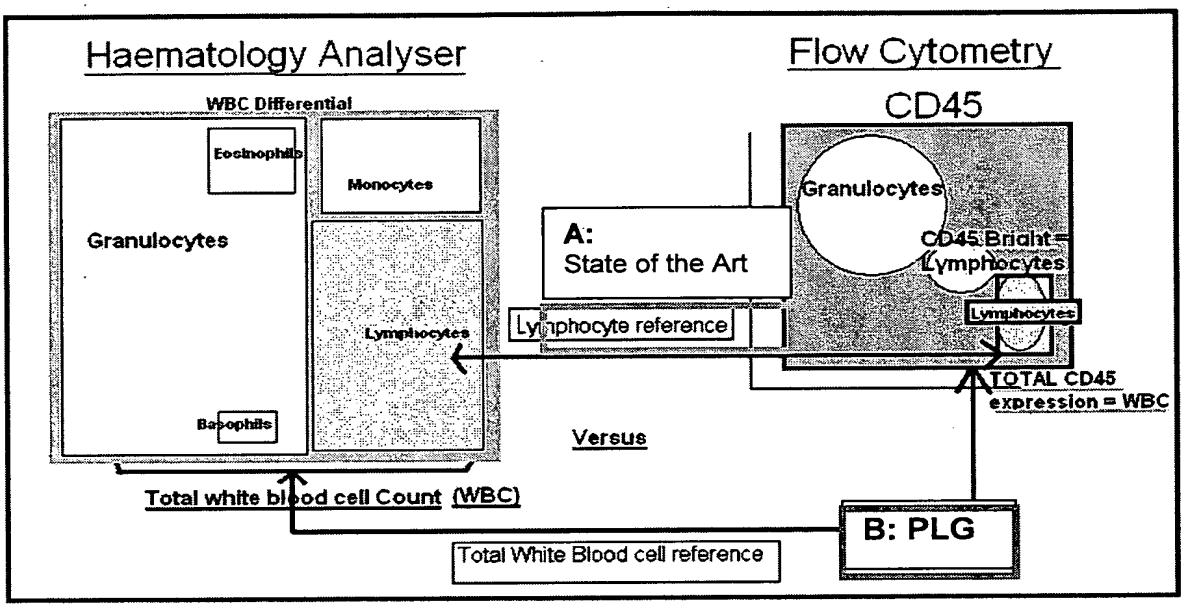
EXHIBIT 1



*PAN*LEUCOGATED (PLG) CD4 COUNTING: A COST EFFECTIVE, SIMPLE AND REPRODUCIBLE SOLUTION FOR HIV/ AIDS MONITORING IN A RESOURCE LIMITED SETTING.

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Costs and delivery of anti-retroviral treatment (ART) and laboratory monitoring for HIV/AIDS represents a significant challenge to any third world country where resources may be limited. In South Africa, as in other countries ravaged by HIV/AIDS, urgent interventions are required to address the high costs and needs for widespread service delivery. Implementation of innovative and sustainable solutions has therefore become critical. It was necessity (as the mother of invention) that drove the development and implementation of PanLeucogated CD4 enumeration testing for HIV/AIDS monitoring in South Africa as an affordable, accessible and robust CD4 testing method.

In April 2003, ~25 new ART support laboratories were established across the South African National Health Laboratory Service (NHLS) to support the South African National HIV/AIDS ART programme. PLG/CD4 testing was chosen as the CD4 method of choice, to be implemented in laboratories in existing regional and new district hospitals, the latter representing a major challenge as the majority of the new sites (all at district level) had no prior knowledge of Flow Cytometry or CD4 testing. An intensive training programme was embarked upon and validation of sites was carried out through collaboration with the Johannesburg NHLS Flow Cytometry reference laboratory. Currently, there is ongoing evaluation of NHLS ART laboratories through participation and performance in the WHO/ NHLS/ QASI Collaborative CD4 Regional External Quality assessment Scheme (REQAS) with 5-6 annual shipments, as well as monthly analysis of internal quality control data. Good reproducibility between ~25 PLG/CD4 NHLS sites has been demonstrated; results of their performance on the CD4 REQAS programme has revealed a consistent %CV $\leq$ 10% between laboratories over 13 trials (compared to the average performance of between 15-25% of the pooled REQAS group of 110 laboratories). Good reproducibility between new PLG/CD4 NHLS sites on the CD4 REQAS programme has further demonstrated that the simplified PLG approach to flow cytometry and the body of technologist experience at district sites can facilitate straight-forward transfer of skills, in spite of little or no previous CD4 flow cytometric experience.

PLG/CD4 has improved accessibility of CD4 testing to more remote areas of South Africa to ensure equitable delivery of ART programmes in both rural and urban clinics in South Africa. Use of total CD45, as the reference component of PLG/CD4 can enable extended period for testing up to 5 days post venesection. This has become an important aspect for extending CD4 laboratory services to more remote areas in South Africa where no laboratory services currently exist. Delivery of samples to district hospital laboratories and innovative use cellular telephone technology for delivery of results has further helped to establish wider PLG/CD4 service.

After logistics analysis and the involvement of the Clinton Foundation in negotiating sustainable pricing for reagents and instrumentation, the South African NHLS standardised on SP PLG CD4. Subsequently "Flow Count Rate" ("FCR" i.e. the constant rate at which known concentrations of

beads are counted in a flow cytometer) has been used across all sites and incorporated in the laboratory data management IT system to identify possible pipette error This system ensures quality control and reproducibility of single tube, SP PLG/CD4 analyses prior to release of results. Furthermore, instrument maintenance monitoring and NHLS standardised PLG protocol set-up (important for standardised aged sample analysis) has been enabled through monitoring inherent qualities of the beads themselves.

Name of session: CD4$^+$ T-cell counting: affordable *vs.* comprehensive methods

Version 1 September 2005

- **Keeney** as chairman will clarify that this session focuses only on flow cytometric methods of CD4$^+$ T-cell counting, and will not cover non-flow methods. Both discussants will disclose any liaisons with industrial partners.

(a) Introduction of the voting system [5 minutes]

(a1) Your country of origin:
(1 answer only)

[1] Greece, Turkey, Cyprus or Israel
[2] Other European Union country
[3] Other European, non-EU member country
[4] US or Canada
[5] Rest of world

(a2) Your profession:
(1 answer only)

[1] Academic, MD or PhD
[2] Academic, other
[3] Other (e.g., laboratory technician)

(b) Introductory questions to CD4$^+$ T-cell counting [10 minutes]

(b1) Does your lab perform CD4$^+$ T-cell enumeration?
(1 answer only)

[1] Yes, on a routine basis
[2] Yes, but not routinely (e.g., research only)
[3] No

- Stratification of answers to questions (a2) to (a4) according to answer to (a1)

(b2) Do you perform CD4$^+$ T-cell counting by flow cytometry?
(1 answer only)

[1] Yes
[2] No, we're using an alternative method

[3] We do not perform $CD4^+$ T-cell counting

(b3) If you're using flow cytometry:
(1 answer only)

[1] We use a single platform method
[2] We use a dual-platform method
[3] Please explain these terms!
[4] We do not perform $CD4^+$ T-cell counting by flow

(b4) If you're using flow cytometry:
(1 answer only)

[1] We use a single color method
[2] We use a 2-color method
[3] We use a 3-color method
[4] We use 4 or more colors
[5] We do not perform $CD4^+$ T-cell counting by flow

*(c) Presentation by **Barnett:** state-of-the-art, 3-/ 4-color ("dual-anchor"), single-platform assay. Data from external quality assurance programmes and published guidelines to support their extensive use. Limitations of single and dual platform methods [15 minutes]*

*(d) Presentation by **Glencross:** description of PanLeucogating method as cost-effective alternative to 4-color ("dual-anchor") methodology. Use of PanLeukogating method in combination with dual- and single-platform absolute counting techniques. Data from external quality assurance programmes to support the position of PanLeukogating [15 minutes]*

(e) The Discussion, and The Vote [15 minutes]
Following the presentations, what is the likelihood of your changing to PanLeucogating
Unlikely
Likely
Very Likely
Not at all

(e1) Your analysis method for $CD4^+$ T-cell enumeration:
(1 answer only)

[1] Dual-anchor gating
[2] Pan*Leuco*gating
[3] None of the above
[4] We do not perform $CD4^+$ T-cell counting by flow

- Stratification of answers to questions (e2) to (e4) according to answer to (e1)

(e2) Your absolute counting method for $CD4^+$ T-cell enumeration:
(1 answer only)

[1] Single platform
[2] Dual platform
[3] None of the above
[4] We do not perform $CD4^+$ T-cell counting by flow

(e3) The use of CD3 as gating reagent in dual-anchor analyses is redundant:
(1 answer only)

[1] Agree
[2] Disagree
[3] No opinion

(e4) Pan*Leuco*gating allows fewer internal controls than dual-anchor gating and is therefore less reliable:
(1 answer only)

[1] Agree
[2] Disagree
[3] No opinion

(e5) For monitoring individual patients, the use of single or dual-platform methods do not make any difference, as long as these methods are not used interchangeably:
(1 answer only)

[1] Agree
[2] Disagree
[3] No opinion

(e6) Will this session impact on your choice $CD4^+$ T-cell counting method?
(1 answer only)

[1] Yes
[2] Maybe
[3] No

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MMWR

Recommendations and Reports

January 31, 2003 / 52(RR02);1-13

Guidelines for Performing Single-Platform A CD4$^+$ T-Cell Determinations with CD45 Gati Persons Infected with Human Immunodeficien

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The material in this report originated in the National Center for Infectious Diseases, James M. Hughes, M.D., Director; Division of AIDS, STD, and TB Labor Kaplan, M.D., Acting Director.

Summary

These guidelines were developed by CDC for laboratorians who perform immunophenotyping for d enumeration of CD4$^+$ T-cells and other lymphocyte subsets in persons infected with human immuno (HIV). The guidelines describe single-platform technology (SPT), a process in which absolute coun subsets are measured from a single tube by a single instrument. SPT incorporates internal calibrat quantity in the analysis of specimens by three- or four-color flow cytometry. With CD45 gating, the of beads and lymphocyte subsets are enumerated, and their absolute numbers and percentage value This report supplements previous recommendations published in 1997 (CDC. 1997 revised guidelin CD4+ T-cell determinations in persons infected with human immunodeficiency virus [HIV]. MMW RR-2]) *that describe dual-platform technology, a method in which absolute counts are derived from obtained from two instruments---a flow cytometer and hematology analyzer. The new recommendat concerns specific to the implementation of SPT as well as other general topics such as laboratory s specimen handling.*

Introduction

Obtaining accurate and reliable measures of CD4$^+$ T lymphocytes (CD4$^+$ T cells) is essential to ass system and managing the health care of persons infected with human immunodeficiency virus (HIV pathogenesis of acquired immunodeficiency syndrome (AIDS) is largely attributable to the decrease

T cells that bear the CD4 receptor (*5--9*). Progressive depletion of $CD4^+$ T cells is associated with a likelihood of severe HIV disease and an unfavorable prognosis (*10--13*). Accordingly, the U.S. Publ (PHS) has recommended that $CD4^+$ T-cell levels be monitored every 3--6 months in all HIV-infecte Measurement of $CD4^+$ T-cell levels has been used to establish decision points for initiating prophyl *Pneumocystis carinii* pneumonia and other opportunistic infections and for initiating and monitorin therapy (*15--20*). $CD4^+$ T-cell levels are also a criterion for categorizing HIV-related clinical condit CDC's classification system for HIV infection and surveillance case definition of AIDS among adul (*21*).

Single-platform technology (SPT) is designed to enable determinations of both absolute and percent subset values using a single tube. Until recently, most absolute T-cell numbers were derived from th measurements determined with two different instruments, a hematology analyzer and a flow cytome technology [DPT]). Hence, the $CD4^+$ T-cell number is the product of three laboratory measurement cell count, the percentage of white blood cells that are lymphocytes (differential), and the percentag that are $CD4^+$ T cells (determined by flow cytometry). In 1997, CDC published guidelines addressi related to DPT (*22*); those guidelines remain appropriate for laboratories performing $CD4^+$ T-cell c technology.

On November 14--15, 2001, a third national conference on $CD4^+$ immunophenotyping was held in to discuss scientific and technologic advances in the development and production of reagents, instru software that have occurred since publication of the 1997 guidelines. The conference was attended b from public health, private, and academic laboratories as well as product manufacturers. These guid consensus of that conference, reviewed by attendees, and specifically related to the performance of

Development of new guidelines was driven by advances in knowledge and experience with new app enumerate $CD4^+$ T cells. First, a gating strategy for identifying lymphocytes using CD45 fluorescen scattering characteristics is now the preferred method for identifying lymphocytes accurately and re Second, three- or four-color flow cytometry has been demonstrated to be superior to two-color meth measuring $CD4^+$ and $CD8^+$ T-cell counts (*23*). Finally, the availability of Food and Drug Administr approved commercial microfluorosphere counting reagents for SPT has resulted in decreased interla variability (*24,25*). Consequently, SPT is the preferred method in an increasing number of laborator

Recommendations

I. Laboratory Safety

A. Use universal precautions with all specimens (*26*).
B. Adhere to the following safety practices (*27--29*):

1. Wear laboratory coats and gloves when processing and analyzing specimens, including specimens on the flow cytometer.
2. Never pipette by mouth. Use safety pipetting devices.
3. Never recap needles. Dispose of needles and syringes in puncture-proof containers desi this purpose.
4. Handle and manipulate specimens (e.g., aliquot, add reagents, vortex, and aspirate) in a biological safety cabinet.
5. Centrifuge specimens in safety carriers.
6. After working with specimens, remove gloves and wash hands with soap and water.
7. For stream-in-air flow cytometers, follow the manufacturer's recommended procedures eliminate the operator's exposure to any aerosols or droplets of sample material.
8. Disinfect flow cytometer wastes. Before adding waste materials to the waste container, sufficient volume of undiluted household bleach (5% sodium hypochlorite) so that the f

concentration of bleach will be 10% (0.5% sodium hypochlorite) when the container is add 100 mL of undiluted bleach to an empty 1,000-mL container).

9. Disinfect the flow cytometer as recommended by the manufacturer. One method is to fl flow cytometer fluidic chambers with a 10% bleach solution for 5--10 minutes at the en day and then flush with water or saline for at least 10 minutes to remove excess bleach, corrosive.
10. Disinfect spills with household bleach or an appropriate dilution of mycobactericidal di Note: Organic matter will reduce the ability of bleach to disinfect infectious agents. NC recommendations regarding how to disinfect specific areas should be followed (*30*). Fo smooth, hard surfaces, a 1% solution of bleach is usually adequate for disinfection; for surfaces, a 10% solution is needed (*30*).
11. Ensure that all samples have been properly fixed after staining and lysing but before an **Note**: Some commercial reagents employ a single-step, lyse and fix method that reduce infectious activity of cell-associated HIV by 3--5 logs (*31,32*); however, these reagents been evaluated for their effectiveness against other agents (e.g., hepatitis virus). Cell-fr be inactivated with 1% paraformaldehyde within 30 minutes (*33--35*).

II. Specimen Collection for Single-Platform Technology

A. Anticoagulant

1. Use tripotassium ethylenediamine tetraacetate (K_3EDTA, 1.5 ± 0.15 mg/mL bloo heparin (*36--39*), and perform the test within the time frame allowed by the SPT manufacturer. Because acid citrate dextrose is added as a liquid to blood collectic tubes, its use would make calculating accurate final sample volume difficult and recommended. With this absolute counting technology, use of an accurate sampl volume is critical.
2. Reject specimens that cannot be processed within 72 hours.

B. Collect blood specimens by venipuncture (*40*) into evacuated tubes containing K_3EDTA anticoagulant, completely expending the vacuum in the tubes.

1. Use pediatric tubes to obtain specimens from children, and ensure that the tube is
2. Mix the blood well with the anticoagulant to prevent clotting.

C. Label all specimens with the date, time of collection, and a unique patient identifier. Ensur patient information and test results are accorded confidentiality.

III. Specimen Transport

A. Maintain and transport specimens at room temperature (64°--72°F [18°--22°C]) (*39,41-* Specimens should not be exposed to extreme temperatures that could allow them to fre become too hot. Temperatures >99°F (37°C) might cause cellular destruction and affec cytometry measurements (*39*). In hot weather, pack the specimen in an insulated contai necessary, place this container inside another containing an ice pack and absorbent mat method helps retain the specimen at ambient temperature. The effect of cool temperatu <39°F [4°C]) on CD45 gate-based immunophenotyping results is not clear (*39,43*).
B. Transport specimens to the immunophenotyping laboratory as soon as possible.
C. For transport to locations outside the collection facility, follow state or local guidelines. method for packaging such specimens is to place the tube containing the specimen in a container (e.g., a sealed plastic bag) and to pack this container inside a cardboard canist containing sufficient material to absorb all the contents should the tube break or leak. C canister tightly. Fasten the request slip securely to the outside of this canister with a rub

For mailing, this canister should be placed inside another canister containing the mailin

D. For interstate shipment, follow federal guidelines for transporting diagnostic specimens at http://www.cdc.gov/od/ohs/biosfty/shipregs.htm). Note: Use overnight carriers with established record of consistent overnight delivery to ensure arrival the following day. these carriers for their specific packaging requirements.
E. Obtain specific protocols and arrange appropriate times of collection and transport from collecting the specimen.

IV. Specimen Integrity

A. Inspect the tube and its contents immediately upon arrival.
B. Take corrective actions if any of the following occur:

1. If the specimen is hot or cold to the touch but not obviously hemolyzed or frozen process it but note the temperature condition on the worksheet and report form. D rapidly warm or chill specimens to bring them to room temperature because this adversely affect the immunophenotyping results (*39*). Abnormalities in light-scat patterns may reveal a compromised specimen.
2. If blood is hemolyzed or frozen, reject the specimen and request another.
3. If clots are visible, reject the specimen and request another.
4. If the specimen is received >72 hours after collection, reject it and request anothe

V. Specimen Processing

A. Perform the test within 48 hours (preferred), but no later than 72 hours after drawing th specimen (*44*).
B. Place the samples on a gentle blood rocker for 5 minutes to ensure that the samples are distributed.
C. Pipette blood volumes accurately and in a reproducible manner. A reverse pipetting tec recommended (Box.
D. Vortex sample tubes to mix the blood and reagents and break up cell aggregates. In add vortex samples immediately after the lyse/fixation step and before analysis to disperse optimally.
E. Incubate all tubes in the dark during the staining procedure.
F. A lyse/no-wash method is required for SPT. Follow directions provided by the manufac
G. Immediately after processing the specimens, cap the tubes and store all stained samples and under refrigeration (39°--50°F [4°--10°C]) until flow cytometric analysis. These sp should not be stored for longer than 24 hours unless the laboratory can demonstrate tha fluorescence patterns do not change for specimens for stored longer periods.

VI. Monoclonal Antibody Panels

A. CD45 is required to aid in the identification of lymphocytes. Lymphocytes are brightly CD45 and have low light-scattering characteristics.
B. Monoclonal antibody panels must contain appropriate antibody combinations to enume and $CD8^+$ T-cells and to ensure the quality of the results (Table 1).

1. CD4 T-cells are identified as being positive for CD3 and CD4.
2. CD8 T-cells are identified as being positive for CD3 and CD8.

C. Three-color monoclonal antibody panels

1. Three-color monoclonal antibody panels should fulfill the following basic

requirements: enumerate $CD4^+$ and $CD8^+$ T-cells, validate the CD45 gate used, a provide some assessment of tube-to-tube variability.

2. Three-color monoclonal antibody panels must consist of at least two tubes, each the same lineage marker. For the examples described previously, CD3 is the com lineage marker in each tube. Differences between replicate CD3 results should be ≤2%.
3. $CD19^+$ B-cell values may be important in assessing immune status of pediatric patients.

D. Four-color monoclonal antibody panels

1. Addition of CD45 to a single tube containing CD3, CD4, and CD8 allows the identification of lymphocytes based on CD45 and side scatter and the enumeratio $CD4^+$ and $CD8^+$ T-lymphocytes.
2. $CD19^+$ B-cell values may be essential for assessing the immune status of pediatri patients.
3. Use of a second tube containing a natural killer (NK) cell marker together with C and CD19 can help to assess the recovery and purity of the lymphocytes within tl CD45/side-scatter gate.

VII. Negative and Positive Controls for Immunophenotyping

Note: An isotype control is not needed.

A. Positive methodologic control

1. Use the methodologic control to determine whether procedures for preparing and processing the specimens are optimal. Prepare this control each time specimens f patients are prepared.
2. Use either a whole-blood specimen from a control donor or commercial materials validated for this purpose.
3. If the methodologic control falls outside established normal ranges, determine the reason. **Note**: The purpose of the methodologic control is to detect problems in preparing and processing the specimens. Biologic factors that cause only the who blood methodologic control to fall outside normal ranges do not invalidate the res from other specimens processed at the same time. Poor lysis or poor labeling in a specimens, including the methodologic control, invalidates results.

B. Positive control for evaluating reagents

1. Use the positive control to test the labeling efficiency of new lots of reagents or v the labeling efficiency of the current lot is questioned. Prepare this control only w needed (i.e., when reagents are in question) in parallel with reagent lots of known acceptable performance. Note: New reagents must demonstrate similar results to of known acceptable performance.
2. Use a whole-blood specimen or other human lymphocyte preparation (e.g., cryopreserved or commercially obtained lyophilized lymphocytes or stabilized w blood).

VIII. Flow Cytometer Quality Control

A. Verify optical alignment daily. Usually, clinical flow cytometers that are capable of thr four-color immunophenotyping have fixed optical systems, i.e., the relative position of cell with respect to the optical elements is fixed. In such systems, the instrument operat

optimize alignment but must verify that the instrument meets the manufacturer's specifi optical alignment. Regardless of whether the alignment is user adjustable, it should be c with alignment standards, such as wide-spectrum fluorescent microfluorospheres with r light-scatter characteristics. Daily monitoring of optical alignment ensures that the cyto acceptably bright fluorescence measurements and that homogeneous peaks are produce parameters to be used in sample analysis (*45*).

1. Use a stable calibration material (e.g., microfluorospheres labeled with fluorochromes) that has measurable and known forward-scatter, side-scatter, and fluorescence properties in each channel to be used for sample analysis.
2. Verify acceptable optical alignment by establishing that calibration particles mee manufacturer- or laboratory-defined criteria for brightness and homogeneity.
3. Align stream-in-air flow cytometers daily (at a minimum) and stream-in-cuvette ı cytometers (most clinical flow cytometers are this type) as recommended by the manufacturer.

B. Standardize fluorescence and light-scatter signals daily. This ensures that the flow cyto operating within manufacturer- or laboratory-defined acceptance ranges under test-spec conditions each day and that its performance is consistent from day to day.

1. Select machine settings that are appropriate for antibody/fluorochrome-labeled, whole-blood specimens.
2. Use microfluorospheres or other stable standardization material to place the scatt and fluorescence peaks in the same narrow range of scatter and fluorescence char each day. Adjust the flow cytometer as needed.
3. Retain machine standardization settings for the remaining quality control procedı (sensitivity and color compensation) and for reading the specimens.

C. Determine fluorescence resolution daily. The flow cytometer must differentiate betwee peak and autofluorescence in each fluorescence channel (*45*).

1. Unstained and lysed fresh whole blood is suitable for adjusting the photomultipli tube (PMT) voltages. The autofluorescence from the unstained lymphocytes shou completely on scale (i.e., <5% of cells within the lymphocyte light-scatter gate fa channel 0 in each fluorescence scale) and should fall within the lower left quadra the dot plot for every PMT/detector in use.
2. Evaluate standardization/calibration material or cells to verify that cells with low fluorescence can be resolved from autofluorescence (e.g., microbeads with low-l and negative fluorescence, CD56-labeled lymphocytes, or dim cells in CD8-label lymphocytes).
3. Establish a minimal acceptable distance between peaks; monitor this difference, a correct any daily deviations.

D. Compensate for spectral overlap daily (Figure 1). Compensation is the process of corre spectral overlap of one fluorochrome into the filter window being used to monitor anotl fluorochrome. In most instruments used clinically, this correction is done by adjusting t electronic compensation circuits on the flow cytometer to place populations not expecte dual positive for two fluorochromes into orthogonal fluorescence quadrants with no ove the double-positive quadrant. At the same time, avoiding overcompensation is essential this may cause dual-positive cells to be incorrectly classified as single positive. The foll procedures may be performed manually, or the software on the flow cytometer may per spectral compensation automatically.

1. Select the compensation control so it will match the brightest specimen signal. U:

either microbead or cellular compensation material containing four populations f three-color immunofluorescence (no fluorescence, phycoerithrin [PE] fluorescen only, fluorescein isothiocyanate [FITC] fluorescence only, and a population that i positive for only the third color) or five populations for four-color (the four descr previously and a population that is positive for only the fourth color).

2. Analyze this material, and adjust the electronic compensation circuits on the flow cytometer to place the fluorescent populations in their respective fluorescence quadrants with no overlap into the double-positive quadrant (Figure 1). With thre fluorochromes, compensation must be carried out in an appropriate sequence: FI PE, and the third color, respectively (*46*). For four-color monoclonal antibody pa follow the flow cytometer manufacturer's instructions for four fluorochromes. A overcompensation.
3. If standardization or calibration particles (microbeads) have been used to set compensation, confirm proper calibration by using lymphocytes labeled with FIT and PE-labeled monoclonal antibodies and a third-color- or fourth-color-labeled monoclonal antibody for three-color or four-color panels, respectively. So that separate cell populations can be recognized without overlap, cells in individual t may be separately stained with each different fluorochrome-labeled antibody and combined in a single tube for analysis. These populations should have the brighte expected signals. Note: Using a dimmer-than-expected signal to set compensatio result in suboptimal compensation for the brightest signal.
4. Reset compensation when photomultiplier tube voltages or optical filters are cha
5. Commercially available software can analyze data without compensation and per the compensation automatically. When using this software, follow manufacturer' instructions for this procedure.

E. Repeat all four instrument quality control procedures (section VIII A--C) whenever ins problems occur or if the instrument has been serviced.
F. Maintain instrument quality control logbooks and monitor them continually for change the parameters. In the logbook, record instrument settings, peak channels, and coefficie variation (CV) values for materials used to monitor or verify optical alignment, standar fluorescence resolution, and spectral compensation. Reestablish target fluorescence lev quality control procedure when lot numbers of beads are changed or the instrument has serviced.

IX. Sample Analyses

A. With single-platform absolute count determination, use of the lyse/no-wash sample pro mandatory. The lymphocyte population is identified as having bright CD45 fluorescenc side-scattering properties (Figure 2). Set the threshold or discriminator as recommende manufacturer. Adjust side scatter so that all leukocyte populations are visible. Draw a g bright $CD45^+$ cell population and analyze the cells in that population (*47*).
B. Count at least 2,500 gated lymphocytes in each sample to ensure that enough cells and l been counted to provide an accurate absolute lymphocyte value.

X. Data Analysis

A. A. CD45 gating

1. Lymphocytes are identified by being brightly labeled with CD45 monoclonal ant and having low side-scattering properties. Two typical examples of a four-color S analysis based on CD45 gating are illustrated (Figure 2).
2. Establish criteria for cluster identification based on a clear definition of lymphoc that does not include basophils (less bright CD45, low side scatter) or monocytes

bright CD45, moderate side scatter). **Note**: Care must be taken to include all lymphocytes. CD45 fluorescence may be slightly less with B cells than with T ce (the major cluster of lymphocytes). NK cells have bright CD45 fluorescence but l slightly more side-scattering properties than the majority of the lymphocytes.

3. CD45/side-scatter gates for lymphocytes are assumed to contain >95% lymphocy Lymphocyte purity is assumed to be high with the CD45/side-scatter gating strat therefore, correction of lymphocyte subset values is not needed (*47*).
4. If an estimate of lymphocyte recovery is needed (i.e., percentage of total lympho within the CD45/side scatter gate), all the B and NK cells must be immunopheno as well. Note: Validation of a CD45/side-scatter gate is recommended during its i use to help determine the CD45 and side-scatter characteristics of T, B, and NK c and to ensure their inclusion in the gate.

B. Set cursors based on the tube containing CD3/CD4 and CD3/CD8 so that the negative a cells in the histogram are clearly separated.
C. Analyze each patient or control specimen with lymphocyte gates and cursors for positiv that particular patient or control.
D. Include the following analytic reliability checks, when available:

1. With SPT, an additional analytical tool can be used to check the accuracy of the absolute count; time can be used as a parameter to determine how long it takes to obtain a microfluorosphere count that represents a unit volume of blood analyzed Optimally, if blood pipetting was performed without noticeable error and the bea were accurately added to the tubes, the time required to analyze a microliter of w blood should be constant. Follow manufacturer's instructions to set time as an act parameter. If more or less time is required for a sample to accumulate the usual number of microspheres, this may indicate a serious counting problem and specir processing should be repeated.
2. Optimally, the sum of the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells shoul equal the total percentage of $CD3^+$ cells $\pm$ 5%, with a maximum variability of <1 **Note:** For specimens containing a considerable number of T $\gamma\delta$ T-cells (*48,49*), t reliability check may exceed the maximum variability.

XI. Data Storage

A. Store list-mode data for all specimens analyzed. This allows for reanalysis of the raw d including redrawing of gates. At a minimum, retain hard copies of the CD45/side-scatte correlated dual-histogram data of each sample's fluorescence.
B. Retain all primary files, worksheets, and report forms for 2 years or as required by state regulation, whichever is longer. Data can be stored electronically. Disposal after the ret period is at the discretion of the laboratory director.

XII. Data Reporting

A. Report all data in terms of CD designation, with a short description of what that design means. **Note:** $CD4^+$ T cells are T-helper cells. The correct cells to report for this value a that are positive for both CD3 and CD4. Similarly, $CD8^+$ T-cells are T-suppressor/cyto and are positive for both CD3 and CD8. Do not include other cell types (non-T cells) in CD8 T-cell determinations.
B. Report lymphocyte subset values as follows:

1. Report both percentages and absolute counts.
2. With SPT, determine the absolute counts directly from the flow cytometers. Thes

calculations are usually handled by software that reports calculated results. The following formula should be used:

$$\frac{\text{No. of events in the bright CD45 region}}{\text{No. of events in the microfluorosphere region}} \times \frac{\text{Total no. of microfluorospheres added}}{\text{Volume of blood added}}$$

C. Report data from all relevant monoclonal antibody combinations with corresponding re limits of expected normal values (e.g., $CD4^+$ T-cell absolute number and percentage). F limits for immunophenotyping test results must be determined for each laboratory (*45*). reference ranges must be established for adults and children, and the appropriate ranges reported for patient specimens.

XIII. Quality Assurance

A. Ensure the overall quality of the laboratory's $CD4^+$ T-cell testing by monitoring and ev effectiveness of the laboratory policies and procedures for the preanalytic, analytic, and postanalytic testing phases. The practices and processes to be monitored and evaluated following:

- methods for collecting, handling, transporting, identifying, processing, and storin specimens;
- information provided on report forms for test requests and results;
- instrument performance, quality control protocols, and maintenance;
- reagent quality control protocols;
- process for reviewing and reporting results.
- employee training and education, which should consist of the following:
  --- basic training by flow cytometer manufacturers and additional training involvi hands-on workshops for flow cytometer operators and supervisors;
  --- education of laboratory directors regarding flow cytometric immunophenotypi through workshops and other programs;
  --- continuing education regarding new developments for all flow cytometric immunophenotyping personnel through meetings and workshops;
  --- adherence to federal and state regulations for training and education;

- assurance of satisfactory performance. Laboratories must fully participate in a performance ev and demonstrate acceptable level of performance. When proficiency testing programs have be the Centers for Medicare & Medicaid Services (formerly, the Health Care Financing Adminis meeting the requirements of the Clinical Laboratory Improvement Amendments of 1988 (CLI currently approved for $CD4^+$ T-cell testing), laboratories must satisfactorily participate.
- review and revision (as necessary or at established intervals) of the laboratory's policies and p ensure adherence to the quality assurance program. All staff involved in the testing should be problems identified during the quality assurance review, and corrective actions should be take recurrences.

B. Document all quality assurance activities.

Evaluation and Validation of a Newly Adopted SPT in the Laborator

When a laboratory adopts the new SPT, specimens should be tested in parallel by using both the cur method to characterize any systematic differences in the methods. Laboratorians should use statistic provide useful information for the comparison studies. Linear least squares regression analyses are l

establishing good correlations between the new and established methods. If no error is detected with the r^2 value will approach 1.0. However, regression-type scatter plots provide inadequate resolution are small in comparison with the analytical range and do not characterize the relationship between t (*50--52*).

A bias scatter plot provides laboratorians with a more useful tool for determining bias. These simple graphs plot the differences in the individual measurements of each method (result of old method---r method) against measurements obtained with one of the methods (result of old method) (*50*). Such easy means of determining if bias is present and distinguishing whether bias is systematic, proportic random/nonconstant. The laboratorian can visually determine the magnitude of these differences ov of values. When sufficient values are plotted, outliers or samples containing interfering substances c The laboratorian can then divide the data into ranges relevant to medical decisions and calculate the (mean of the bias) and the random error (standard deviation of the bias) to gain insight into analytic the specified decision points (*50--52*).

Several detailed guidelines and texts provide additional information regarding quality goals, metho estimation of bias, and bias scatter plots (*50--54*). Once a new method is accepted and implemented will need to confirm or redefine its normal range and should continue to monitor the correlation bet and the patient's clinical disease data to ensure that no problems have gone undetected by the relativ typically tested during method evaluations.

Discussion

More than 1.6 million $CD4^+$ T-cell measurements are performed yearly by the approximately 600 te in the United States (*55*). This figure is based on the reported number of tests performed annually b participating in CDC's Model Performance Evaluation Program (MPEP) for T-lymphocyte immuno 1996. These measurements are performed with flow cytometers using either multiplatform technolo was introduced for clinical application in 1996, and its wide-scale implementation is relatively new. of two independent multicenter studies studies of SPT were reported (*24,25*). Those and subsequent and CD45 gating (*56--60*) have increasingly encouraged adoption of these improved testing practice resulting outcomes associated with SPT and CD45 gating include a) increased confidence in results, reproducible results, c) increased ability to resolve discrepant problems, d) decreased proportion of specimens received for testing, e) decreased proportion of specimens requiring reanalysis, and f) fe could pose biohazard risks (*61*).

Although these guidelines for SPT use might foster improved laboratory practices, developing com guidelines for every aspect of $CD4^+$ T-cell testing (including some laboratory-specific practices) is Moreover, measuring the outcomes associated with the adoption of these guidelines is inherently di guidelines lack evaluation protocols that can adequately account for the interactions among the reco weight of importance has been assigned for the individual recommendations that address unique ste process; hence, the consequences of incompletely following the entire set of recommendations are u because published data are not available for every aspect of the guidelines, certain recommendation experience and opinion of knowledgeable persons. Recommendations made on this basis, in the abs be biased and inaccurate. Finally, variations in testing practices and interactions among the practice specimens are obtained and processed, skill of laboratory personnel [such as with pipetting], testing test-result reporting practices, and compliance with other voluntary standards and laboratory regulat both the development of guidelines that will fit every laboratory's unique circumstances and the ass value of implementing the guidelines.

The first CDC recommendations for laboratory performance of $CD4^+$ T-cell testing (*63*) were writte impede development of new technology or investigations into better ways to assess the status of the in HIV-infected persons. Developments in the technology have resulted in an assay that is technical complicated and more accurate. These single-platform methods are now being implemented in as m

of the laboratories in the United States (MPEP data). In addition, other T-cell phenotypic markers ai investigated as prognostic indicators or markers of treatment efficacy, alone and in combination wit markers (*64,65*).

These guidelines for SPT are intended for domestic implementation. Several alternative methods ar require fewer reagents and involve more cost-effective gating algorithms. Some of these alternative compatible with current U.S. clinical laboratory methods; however, to date they have not been valid applications. As published validation data accumulate from multisite studies for methods such as Pa (*66*) and primary CD4 gating (*67, 68*), these potentially more cost-effective options will be consider or substitute methods. In the future, guidelines should be harmonized to include all methods that me performance standards to ensure consistent high quality.

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Table 1

TABLE 1. Three- and four-color monoclonal antibody panels

Panel	Monoclonal antibodies	Notes
Three-color panel	CD3/CD4/CD45*	Gate using CD45 and side scatter; measure CD3+/CD4+ T cells and CD
	CD3/CD8/CD45*	Gate using CD45 and side scatter; measure CD3+/CD8+ T cells and CD
	CD3/CD19/CD45†	Gate using CD45 and side scatter; measure CD3+ T cells and CD19+ B
Four-color panel	CD45/CD3/CD4/CD8§	Gate using CD45 and side scatter; measure CD3+/CD4+ T cells, CD3+/CD8 T cells, and CD3+ T cells
	CD45/CD3/CD19/CD16 and/or CD56†	Gate using CD45 and side scatter; measure CD19+ B cells, CD3+ T cell CD3-/CD16 and/or CD56+ NK¶ cells

* Recommended three-color panels.
† Recommended for specimens obtained from children.
§ Recommended four-color panel.
¶ Natural killer.

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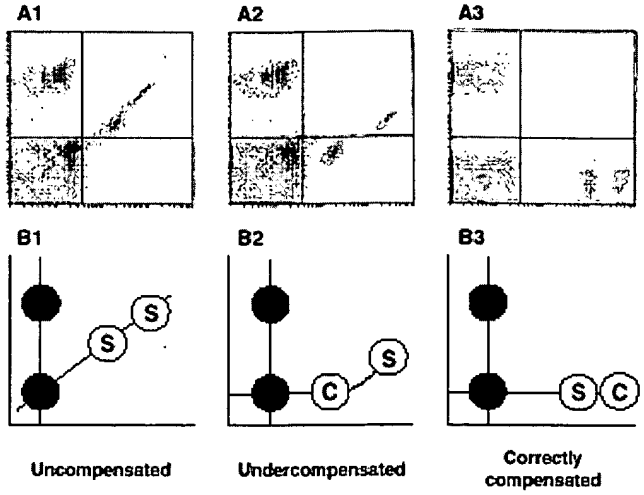
Figure 1

FIGURE 1. The multicolor compensation concept



Histograms A1–A3 and their respective schematic depictions, B1–B3, illustrate uncompensated, undercompensated, and correctly compensated two-color displays, respectively. The solid circles represent the cell populations for which accurate color compensation is desired. The open circles labeled C represent the compensation controls being used to adjust color compensation settings. If the intensity of the color compensation control does not exceed the fluorescence intensity of the sample to be analyzed, that sample will be undercompensated (**A2, B2**). If the intensity of the color compensation control exceeds the fluorescence intensity of the sample to be analyzed, the sample is less likely to be undercompensated (**A3, B3**).

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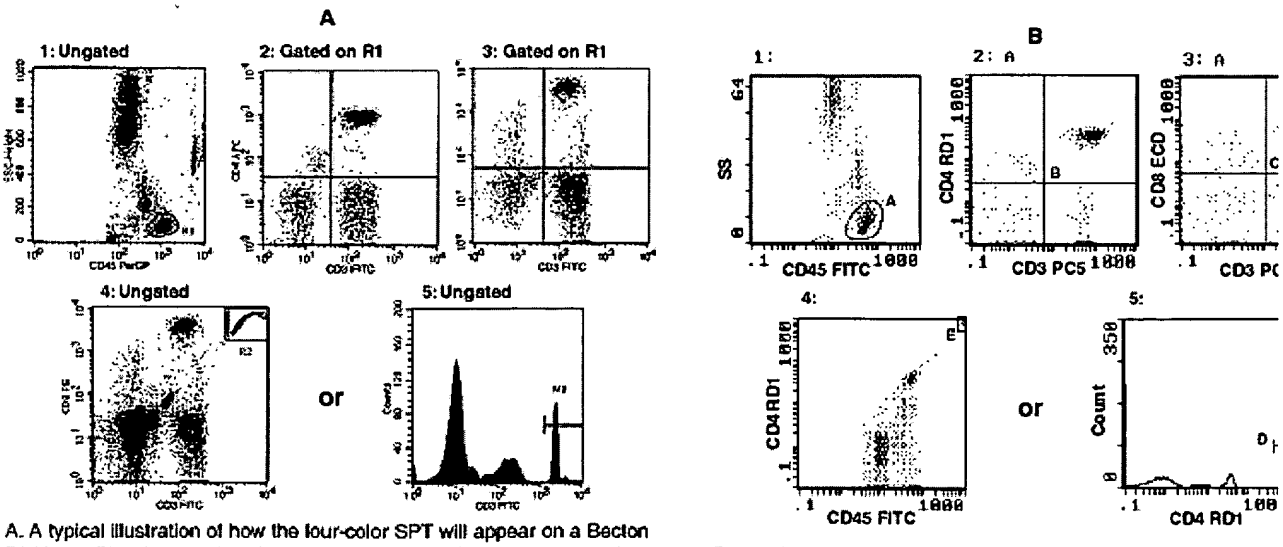
Figure 2

FIGURE 2. Results of single-platform technology (SPT) performed by using CD45 gating with four colors



A. A typical illustration of how the four-color SPT will appear on a Becton Dickinson Biosciences (San Jose, CA) FACSCalibur™ instrument monitor. Dot plots 1–3 illustrate the CD45 versus side-scattering, the CD4 versus CD3, and the CD8 versus CD3 dot plots, respectively. Graphs 4 and 5 illustrate the two options available to monitor the presence of the microfluorospheres used for absolute cell counting calculations. The microfluorospheres in graphs 4 and 5 are located in regions R2 and M1, respectively.

B. A typical example of the four-color SPT a on Beckman Coulter FL) Epics XL™ instrument monitor. Dot plots 1–3 illustrate th versus side scatter, the CD4 versus CD3, and the CD8 versus plots, respectively. Graphs 4 and 5 illustrate the two options ava monitor the presence of the microfluorospheres. The microfluoro in graphs 4 and 5 are located in regions E and D, respectively.

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Box

BOX. Accurate pipetting technique

Use of SPT to obtain absolute CD4 counts requires accurate and precise measurement of blood and beads. Reverse pipetting technique is recommended for dispensing these products.

Testing Pipetting Precision

The precision of pipetting should be evaluated periodically (e.g., monthly) to ensure the accuracy of results. Retain all records of this evaluation procedure for quality assurance purposes.

- Using the reverse pipetting technique, pipette 10 replicates of blood and record the weights. Select a volume normally used in the performance of the assay.
- Using the reverse pipetting technique, pipette 10 replicates of bead suspension and record the weights (this applies to methods in which the beads must be pipetted into the tubes).
- Calculate the mean, standard deviation, and coefficient of variation (CV). The CV for replicates should be <2% (Table 2).

Testing Pipetting Accuracy

The following procedure can be used to test the pipette and how accurately it measures volume. Water is used because the weight of 1 µL of water is 1 µg.

- Using the reverse pipetting technique, pipette 10 replicates of distilled water and record the weight. (100 µL of water should weigh 0.1000 grams.) (Table 2)
- Calculate the mean, standard deviation, and CV. The CV must be <2% (range: 0.098-0.102).

Procedures

The following information is consolidated from operational instruction manuals from several pipette manufacturers. Complete information and more detailed instructions are contained in specific pipette instruction manuals; some of these are available online. Read the manufacturer's manual carefully before beginning the pipetting procedure.

- Select the desired volume (with manual pipettes, higher volumes should be set first; if adjusting from a lower to a higher volume, first surpass the desired volume and then slowly decrease the volume until the required setting is reached).
- If applicable, select the desired mode (e.g., reverse pipette). This is recommended for optimal precision and reproducibility.
- Reverse pipetting can be done with a manual pipette by pressing the control button slightly past the first stop when aspirating, taking up more liquid than will be dispensed, then pressing the control button only to the first stop when dispensing. A small volume will remain in the tip after dispensing.
- Select an appropriate tip (usually color matches the color of the control button).

Prerinsing

The following procedures will help ensure optimal precision and accuracy.

- Volumes >10 µL: Prerinse pipette 2–3 tim
  (this involves aspirating and dispensing li
  Reasons for prerinsing include the follow
  —to compensate for system pressure, for sl
  temperature between pipette and liqu
  ties of the liquid;
  —to clear the thin film formed by the liqui
  the pipette. Without prerinsing, reter
  on the inside wall of the tip would ca
  to be too small. The thickness and nat
  therefore the potential source of error,
  on the nature of the liquid being pipe
- Volumes <10 µL: Do not prerinse pipette
  dispensing to ensure that the whole volt
  For smaller volumes, prerinsing is not rec
  the dispensed volume would be too great.

Filling

- Make sure tip is securely attached.
- Hold pipette upright.
- When aspirating, try to keep the tip at a co
  the surface of the liquid.
- Glide control button slowly and smoothly
  perform this step automatically).
- When pipetting viscous liquids (e.g., who
  tip in the liquid for 1–2 seconds after aspi
  drawing it.
- After liquid is in the tip, never lay the pip

Dispensing

- Hold the tip at an angle, against the insid
  tube if possible.
- Glide control button slowly and smoothly
  perform this step automatically).

Other Recommendations

- To ensure optimal performance, the te
  pipetted solution and the pipette and tips
  (volume errors may occur because of chan
  ment and viscosity of the liquid). Do not
  temperatures >70°C.
- Volume errors may also occur with liquic
  vapor pressure or a density/viscosity that
  water. Water is most commonly used to ca
  to check inaccuracy and imprecision. A pi
  be recalibrated for liquids with densities th
  that of water.
- Pipettes should be checked regularly for pre
- Regular maintenance (e.g., cleaning) sh
  either by the user or a service techni
  manufacturer's instructions.

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Procedural guidelines for performing immunophenotyping by flow cytometry.

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Flow cytometry is a rapidly expanding technology that is moving from the research laboratory into the clinical laboratory. Recent advances in availability and reproducibility of monoclonal antibody reagents specific for a wide range of cell types coupled with lower costs for increasingly automated flow cytometers with powerful and user friendly data analysis capabilities have made flow cytometry the method of choice for immunophenotyping in the clinical laboratory. However, there is great variability in the level and type of quality assurance procedures used from laboratory to laboratory. A subcommittee established by the National Committee for Clinical Laboratory Standards (NCCLS), composed of representatives from industry, academia, professional societies, and regulatory agencies, has drafted consensus procedures which address specific problems and suggested solutions for performance of immunophenotyping by flow cytometry. This paper is based on the authors' discussions with the NCCLS Committee but does not represent an official NCCLS position. The official NCCLS document on this subject (H42) is expected to be published in 1989.

Publication Types:
- Review
- Review, Tutorial

PMID: 2656019 [PubMed - indexed for MEDLINE]

Performance of PanLeucogated (PLG) CD4 lymphocyte enumeration: Cross platform equivalency, accuracy and reproducibility versus state of the art CD4 methods.

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ABSTRACT

To come.

Acknowledgements: *This study was supported in South Africa, in part by NHLS Department of Molecular Medicine and Haematology R&D Fund, as well as NIH funded Johannesburg CIPRA grant Core B Project and Project V. We thank Dr. Daniella Livnat, DAIDS, NIH, for ongoing support and valuable criticisms of PLG/CD4 protocols, as well as Dr. Mike Keeney for comments and criticism of the manuscript.*

We thank the NICD, National Health Laboratory Service, Johannesburg, viz. Dr A Puren and Moses ..... for use of FACSCount results.

The authors would like to acknowledge Dr Gaby Vercauteren from the Division of Blood Safety, World Health Organisation (WHO), Geneva and Dr. Frank Mandy, Health Canada, Quality Assessment Scheme Immunology (QASI) for their respective contributions and support of the WHO/ NHLS/ QASI CD4 Regional External Quality Assessment Scheme (REQAS).

Interests*: The South NHLS is the holder of the PLG/CD4 enumeration patent, licensed to BCI for worldwide affordable CD4 enumeration. FlowCARE PLG/CD4 received FDA 510(k) approval in January 2005.*

INTRODUCTION:

Dual Platform (DP) PanLeucogating (PLG) has been introduced as an alternative method to enumerate CD4 Lymphocytes (1) and later shown to be applicable to Single Platform (SP) testing, both by volumetry (2) and

flow cytometric flow rate calibration measurement (3). PLG/CD4, as a method for CD4 testing, utilizes total white blood cells as the reference point for CD4 cell determination. It therefore represents a paradigm shift in primary reference or gating versus conventionally methods that prescribe to use of Total Lymphocytes as the point of reference/ primary gate. In addition, this method has moved away from traditional methodology by defining CD4 Lymphocytes directly without use of CD3 to firstly identify T cells. It relies instead, on flow cytometric physical side scatter properties to separately count CD4 lymphocytes and exclude monocytes from the analysis. The result is a cost effective CD4 method (utilizing only two monoclonal antibodies, as opposed to ~8/9 prescribed by various North American (4, 5) and European groups (6). It is a simpler gating strategy that can facilitate easy transfer of skills to laboratory personnel with no prior skills (7) and can potentially improve reproducibility of CD4 counts between laboratories (8).

During May 2004, the South African National Health Laboratory Service (NHLS) launched its National CD4 enumeration service in support of the South African Department of Health Anti-Retroviral Treatment Programme. PLG (PanLeucogated)/CD4 methodology was chosen through tender process, as the CD4 method for NHLS implementation. A decision was taken by the governing board of the NHLS to implement SP PLG/CD4 across 22 sites, including both district and regional level laboratories. Factors contributing to the choice of SP technology included significant price reduction of reagents, including beads, brokered with the Clinton HIV/AIDS Initiative, as well as logistical issues relating to access to haematology parameters at some sites. The implementation of PLG/CD4 would ensure that costs were kept low and Flow Cytometry concepts (PLG) simple enough to implement at district hospital level, where minimal or no Flow Cytometry skills existed. Further, use of SP PLG/CD4 could facilitate an extended window of testing to ensure that samples transported into districts from very remote sites would still be viable for testing (12 and unpublished data, Glencross and Forman). Prior to this implementation, it was therefore necessary to validate SP PLG/CD4 from the previously validated DP format.

For the purposes of this paper, we have analysed data collected in both Johannesburg and in the US, part of which was presented for the PLG/CD4 US Food and Drug Administration (FDA) 510(k) application by Beckman Coulter International (BC)). Collectively, these analyses include (1) assessment of the cross-platform (DP versus SP) equivalency comparing SP to DP PLG/CD4 and (2) comparison of the accuracy of PLG/CD4 (in either DP or SP format) versus various state of the art CD4 systems. Lastly, we present an analysis of accuracy and reproducibility between laboratories performing PLG/CD4 within the NHLS and other African sites taken from data obtained from the Johannesburg World Health Organisation (WHO)/ NHLS/ QASI collaborative CD4 Regional External Quality Assessment Scheme (REQAS) coordinated in Johannesburg.

MATERIALS AND METHODS

Clinical Samples

The clinical samples used were accumulated from several separate studies, details of which are outlined in Table 1. In summary, the Johannesburg study group comprised a total of 495 fresh routine K_3EDTA blood samples referred for routine CD4 T cell enumeration to the Johannesburg Hospital Flow Cytometry laboratory. The US study comprised 389 clinical EDTA samples (n=48 [BCI, Miami and, FDA 510[k] data n=297 collected across 3 geographical separate US sites). Ethical clearance for testing of these samples in Johannesburg was obtained from the University of the Witwatersrand, Faculty of Health Sciences *Committee for Research on Human Subjects: Clearance Certificate Protocol Number: M00/08/09.* Blood was stored at 18-25°C throughout the study and throughout sites. All details pertaining to these studies, the Mean and Median CD4 counts, the ranges of CD4 counts within these studies and reagents and equipment used throughout respective studies, are outlined in Table 1. Details of how separate study groups were combined for simplification of analysis and presentation of the data are shown comparative study (e.g. the SP PLG/CD4 versus DP PLG/CD4 combined data from both Johannesburg and the US and combined together several data sets including A, B, C, D and E).

White Blood Cell (WBC) counting and quality control

The White cell counts (WCC) required for DP PLG/CD4 counting were measured using a GenS or STKS haematology instrument (both BCI, Miami, FL). Daily appropriate internal quality control was performed to ensure both accuracy and precision of all haematology instruments used throughout sites. All samples were analyzed once to obtain a white cell count (WCC), which was subsequently used for the calculation of relevant DP PLG/CD4 results (see Figure 1 for details of how the WCC is used in the DP PLG/CD4 calculation).

Sample preparation

One hundred microlitres (ul) aliquots of patient blood were added to appropriately labeled test tubes and vortexed once. For all PLG/CD4 preparation, anti CD45FITC and CD4PE reagents (FlowCARE PLG/CD4 reagents, BCI, Miami, FL) were used as prescribed by the manufacturer (Table 1) and the sample incubated for 15-minutes in the dark. For all "Predicate"/ state of the art 4-colour CD4 testing, TetraONE reagents (CD8FITC/ CD4PE, CD45ECD and CD3PerCP) were used in accordance with the suppliers' recommendations (BCI, Miami, FL). For all samples, the whole blood lysis system (BC QPrep /ImmunoPrep™, Miami, FL, USA) was used throughout the study, to lyse red blood cells, to stabilize and fix the samples. For all SP testing (irrespective of use of PLG/CD4 or "Predicate" gating strategy), 100 uls of FlowCOUNT ™ beads (BCI, Miami, FL, USA) were added just prior to flow cytometric analysis. Samples that were analysed by FACSCount™ analysis were prepared and analysed as recommended by the supplier (Becton Dickinson Immunocytometry Systems -BDIS, San Hose, CA) at the National Centre for Communicable Diseases, in Johannesburg, with the permission of Dr. A Puren.

"Predicate" versus PLG/CD4 gating.

Throughout the study as previously described (2, 3), CD45*bright* gating of lymphocytes, with subsequent CD3+ gating to identify CD3+/CD4+ lymphocytes is referred to as "Predicate" and is used in the comparative analyses

both SP or DP format. SP PLG/CD4 gating is used as shown in Figure 3. DP PLG/CD4 gating strategy is used as shown in Figure 1, but without the use of beads noted in Figure 1 "FC Beads". Instead, CD4% of total White Blood cells (CD4%PLG) is used multiplied by the corresponding WCC obtained from a haematology analyser to obtain a DP PLG/CD4 absolute CD4 count.

Flow Cytometry

All samples were analysed either on an XL-MCL or FC500 Flow Cytometer (both BCI, Miami, FL), as indicated in various sub-studies (see Table 1 for details). BC CXP software was used for the analysis of samples analysed by FC500. Daily Internal Quality Control on all flow cytometers was performed using FlowCHECK™ standardised micro-beads to assess linearity and instrument performance respectively. In Johannesburg, system performance was verified daily using with stabilized whole blood (ImmunoTrol™ BC). Listmode files were stored on all samples in order to retrospectively re-analyze outliers in all sites. Through out the study for the purposes of PLG/CD4 analysis, FS threshold was used, as recommended by the supplier (see Figure 1). A FACSCount™ dedicated flow cytometer (BDIS, San Hose, CA) was used for analysis of samples in an additional study (N=74), with appropriate daily quality control and calibration performed as recommended by the supplier.

Intra-laboratory precision testing: intra-laboratory FlowCOUNT™/Bead reproducibility to ensure accuracy and precision of SP PLG/CD4 counting.

The reproducibility of the manual pipetting of beads into separate samples, by a single operator in the Johannesburg laboratory, was assessed (i.e. 100 ul of FlowCOUNT™ beads was manually added to repeated 100 ul aliquots of the same blood sample, ultimately comprising 10 replicates and analysed on a BC XL Flow Cytometer). The Mean, SD and %CV of absolute counts and bead count (FlowCOUNT™) rates were calculated and are presented in Table 2. The flow cytometric bead rate, termed "Flow Count Rate/ FCR" of the FlowCOUNT™ beads, calculated per sample, was used as an additional quality control parameter to exclude manual pipetting error (4) in Johannesburg.

Laboratory Accreditation and External Quality Assessment programme participation

The Johannesburg Hospital Flow Cytometry and Haematology laboratories, under the current auspices of the South African National Health Laboratory Service (NHLS), have accreditation with the South African National Accreditation Scheme (SANAS), with reciprocity to various international accreditation bodies. The Johannesburg Flow Cytometry laboratory initiated and is responsible for the co-ordination of the NHLS/ WHO/ QASI REQAS as well as the NHLS Hematology External Quality Assessment programme. The Johannesburg laboratory participates in the U.K. NEQAS External Quality Assessment Immune Monitoring scheme, the BC Inter-laboratory Quality Assurance Program and the WHO CD4 REQAS. The NHLS sister Haematology laboratory participates in the Royal College Pathologists of Australia (RCPA) Hematology Scheme and NHLS Haematology EQA programme.

Statistical analysis.

To simplify the overall analysis of the data, sets of data from different studies and sites, were combined according to relevant comparative analysis. Linear regression analysis (equation of the line and R^2) and correlation studies (Pearson correlation coefficient r) and Bland-Altman (BA) analysis [10] was used to compare performance between methodologies. The average absolute difference between the two methods, referred to as the bias, as well as the limits of agreements of the BA analysis (LOA = mean difference ± 2 standard deviations) are reported. In all Bland Altman analyses, SP "Predicate" CD4 was used as the standard unless otherwise stated and is always represented the standard minus the test method on the vertical axis of the difference plot. On the horizontal axis of these BA difference plots, the average between the two methods (standard and test method) was used as recommended by Bland and Altman [11]. To simplify the Bland Altman analysis and assess possible differences in the clinically relevant ranges, three separate analyses were performed i.e. (1) overall (all data points across the entire spectrum of the data set including very low CD4 counts < than 50 cells/ul and very high CD4 counts > than 1000 cells/ul) (2) CD4 counts less than 500 cells/ul and (3) and a separate analysis looking at the range of 150-250 CD4 cells/ul.

The Percentage Similarity model designed for multiple method comparison specifically for data with broad ranges [12] was also applied, with the mean percentage similarity (MPS), the Standard Deviation of the MPS (MPS±SD) and the percentage similarity CV (MPS%CV) per data set. The percentage similarity CV shows the overall accuracy and precision between methods. Here SP "Predicate" CD4 was used as the standard in the model formula for comparison in majority of studies, with the exception that DP PLG/CD4 was used as the standard in the SP versus DP PLG/CD4 comparison and FACSCount™ was used as the standard in the FACSCount™ comparisons. Throughout the applications of both models, no outliers were removed. *To simply reading and help the reader to follow the many method comparisons presented in this paper, the test method is always mentioned first and the standard mentioned second, in all text, tables and figures.*

CD4 REQAS analysis of performance of laboratories using PLG/CD4

The inter-laboratory reproducibility and performance of laboratories performing PLG/CD4 participating in the WHO/ NHLS/ QASI CD4 REQAS programme was reviewed and compared to the performance of laboratories performing other types of CD4 technology (as a reference point for comparison of PLG/CD4 laboratory performance). Absolute CD4 results submitted by participating laboratories during 2002-2004, over 8 trials were analysed per trial to derive the Trimmed Mean (excluding outlying laboratories that fell more than 2 SD outside of the initial Pooled Mean analysis), the Standard Deviation (SD) of the Trimmed Mean and percentage co-efficient of variation (%CV). Using these latter values, a Standard Deviation Index (SDI, as a measurement of accuracy of CD4 reporting and giving an idea of the respective laboratory's performance by indicating how close the particular laboratory's results were to the Trimmed Mean result as a function of the number of SD away from the Trimmed Mean) was calculated for each laboratory, per trial The SDI is calculateduses the respective laboratory's residual (the laboratory CD4 result minus Trimmed mean) divided by the Trimmed SD

to derive a laboratory's respective SDI (also referred to as a statistical *z-score*). The SDI results of 8 trials were then pooled and sorted according to technology type. The Mean, the SD and a %CV of the SDI values were calculated within CD4 technology types. Only technologies and methodologies relevant to this publication are included in the analysis i.e. the "Predicate" state of the art-algorithm based system (CD8/4/45/3 Trucount™/ MultiSET™, BDIS, San Hose, CA), the industry SP equivalent system - FlowCOUNT™ beads (BCI, Miami, FL), conventional lymphocyte based DP CD4 assays and the dedicated flow cytometric CD4 counter FACSCount™ (BCI, Miami, FL).

An Outlier rate (OR) was also calculated per CD4 technology group by dividing the difference of N in the Pooled and the Trimmed analysis (which excludes laboratories that fall more than 2SD outside of the Mean) by the total number of laboratories analysed (all laboratories in the pooled analysis). See Table 9 for details.

RESULTS

Evaluation of Cross Platform equivalency of CD4 enumeration: DP PLG/CD4 vs. SP PLG/CD4.

This analysis comprised data sets A, B, C, D and E (see Table 1, N=811), comparing absolute CD4 counts generated by DP PLG/CD4 and SP PLG/CD4 respectively. Results of this statistical analysis are shown in Table 4. Bland Altman analysis (see Figure 2) performed in the clinically relevant range less than 500 (N=481), and within the range of 150-250 CD4 cells/ul revealed a minimal bias between DP versus SP PLG/CD4 of +4.6±17.5 cells/uL, increasing slightly within the 150 -250 cell range to +6.7 cells. The reason for the 2 cell/ul change in bias in the 200 cells/ul is probably related to statistical bias; however the confidence interval and 95% limits of agreement remained relatively constant in both <than 500 and around 200 cells/ul data sets. The Percentage similarity statistics were performed across the entire data set (N=811) as well as in subsets of <than 500 cells/ul (N=481) and in the 150-250 cells/ul range (N=112). This revealed a remarkably constant MPS of ~98.8±4.4% i.e. SP PLG/CD4 reads on average 1±4% lower than DP PLG/CD4 (see Table 4 for details).

Evaluation of Cross Platform equivalency of CD4 enumeration: DP "Predicate" vs. SP "Predicate".

These studies were performed to get an idea how the known state of the art performed when compared to the DP vs. SP PLG/CD4 analysis. Here the analysis comprised data sets A, B and C (see Table 1, N=389) with Table 5 outlining details of this analysis. Overall there was a higher bias between DP and SP "Predicate"CD4 results than comparable DP vs SP PLG/CD4 results. In the <than 500 CD4 cells/ul range, a negative bias of 28 cells/ul was noted, reducing very slightly to 25 cells in the 150-250 CD4 cells/ul range. Overall, Percentage Similarity analysis supported the Bland Altman analysis and revealed that DP Predicate reads on average 5-7±6% higher than Predicate SP CD4 enumeration across the whole data set.

Comparison of accuracy: DP PLG/CD4 versus SP "Predicate" CD4 enumeration.

This analysis comprised data sets A, B and C (see Table 1, N=389), comparing absolute CD4 counts generated by DP PLG/CD4 to SP "Predicate" CD4 counts. Results of this statistical analysis are shown in Table 6. In the clinically relevant range of less than 500 CD4 cells/ul (N=227), Bland Altman analyses revealed a bias -23±30

cells/ul with the 95% limits of agreement between –83 and 35. The Percentage similarity statistics were performed across the entire data set revealed a MPS of 105±6 % i.e. SP "Predicate" CD4 testing reading an average of 3-5% lower than DP PLG/CD4. In the clinically relevant CD4 cell ranges, there was less bias; an 11±23 CD4 cell/ul bias was noted in the 150- 200 cells/ul range.

Comparison of accuracy: SP PLG/CD4 versus SP "Predicate" CD4 enumeration.

This analysis comprised data sets A, B and C (see Table 1, N=389), comparing absolute CD4 counts generated by SP PLG/CD4 to SP "Predicate" CD4 counts. Results of this statistical analysis are shown in Table 7. Bland Altman analyses revealed a bias of -53 overall, reducing to -18 cells/ul in the data set < than 500 and -13 cells/ul in the range around 200 CD4cells cells/ul respectively. MPS analysis showed a 3- 4% difference between results i.e. SP PLG reads on average 3-4% higher than SP "Predicate" CD4 testing.

Comparison of PLG/CD4 and "Predicate" CD4 to FACSCount™.

Three separate analyses were performed (Table 8). On the first FACSCount data set (n=33), DP PLG/CD4 results were compared to FACSCount™ CD4 results (as the standard). The second data set compared SP PLG/CD4 to FACSCount™. Very little difference was noted between PLG/CD4 and FACSCount, in either DP or SP format. In the DP PLG/CD4 comparison, a mean bias of 6-7 cells/ul was noted in the therapeutic decision making range of 200 cells/ul (the median CD4 count in this data set was noted to be 223 cells/ul). Three outliers were noted on this BA analysis; 2 of the latter had CD4 cell counts greater than 800 CD4 cells/ul; the remaining outlier showed a negative bias of 93 cells, with FACSCount reading lower than DP PLG/CD4 (249 vs.342 CD4 cells/ul FACSCount vs. PLG/CD4 respectively). No obvious reason could be established for this latter outlier. To get an idea of the equivalency of "Predicate" CD4 i.e.CD45*bright*, CD3/4+ an additional comparison was also performed to assess similarity between SP "Predicate" and FACSCount™ CD4 results (N=40); (see Table 8 for details of Bland Altman and Mean Percentage Similarity (MPS) statistics). A higher bias of ~44 cells/ ul was noted in this comparison indicating that on average, FACSCount™ reads 44 cells/ul higher than SP "Predicate" (Figure 3). A single outlier was noted to be in the range > than 1500 CD4 cells/ul. Bead reproducibility as assessed by bead FCR on this data set [see Table 1, data set G] was assessed to the influence of pipette error on the outlying results. A %co-efficient of variation (%CV) of the mean bead rate on both SP PLG/CD4 and SP predicate CD4 was noted to be 5.1% and within acceptable limits to exclude pipette error (11).

Comparison of CD4 Percentage of Lymphocytes (CD4%L) in. PLG/CD4 versus "Predicate" systems.

We compared the results of CD4%L generated within the PLG/CD4 protocol using only CD4 expression and side scatter (without CD3) i.e. identifying CD4 bright/ low side scatter cells (figure 1) versus CD4%L performed in "Predicate" testing that utilises bright CD45 expression with CD3 gating to define CD4+ expressing lymphocytes. Table 3 outlines the comparative analyses including Regression analysis/ Correlation, Bland Altman and Percentage Similarity statistics analysing collective data sets (A, B and C, see Table 1 for details). Very little difference (MPS revealed an overall 1% difference between methods with a BA bias of 0.9±1.4%)

between results was noted between CD4%L generated using CD45*bright* expression with CD3/CD4+ versus CD45*bright* with CD4 and SS expression only (Figure 4). Although several outliers were noted on the BA analysis (N=16 of 389), the mean bias of these outliers was –0.2% and the average difference of CD4%L in the outlier group did not vary by more 3% (range –4.4 to +4.4%). Seven outliers were noted on the percentage similarity analysis and were noted to be the same outliers noted on the BA analysis. These results suggest that differential (bright) CD4 expression with SS is sufficient to define CD4+ expressing lymphocytes obviating the need for CD3 gating in defining CD4 lymphocytes. To assess whether there was an influence of instrument itself on the measurement, a comparison of predicate to predicate using XL-MCL CD4%L vs. FC500 CD4%L data (Data set A and B, see Table 1 for details) was performed, using data set A as the standard i.e. BC XL-MCL CD4%L. A similar bias to that noted in the PLG/CD4 comparison was noted with predicate FC500 CD4%L reading 0.9% higher on average than Predicate XL-MCL CD4%L. As expected, when "Predicate CD4%L" was compared to "Predicate CD4%L", both generated on an XL-MCL, a MPS of 100% and a negligible bias of 0.03% was noted.CD4 REQAS analysis of performance of laboratories using PLG/CD4

CD4 REQAS analysis of performance of laboratories using PLG/CD4

Overall the findings of the analysis of the CD4 REQAS confirmed the findings of the direct comparisons performed for this paper. Excellent reproducibility between the laboratories performing PLG/CD4 testing was noted. The mean overall measurement of precision and accuracy as indicated by the %CV between laboratories and the SDI Mean±SD revealed that PLG/CD4 Laboratories and laboratories that use FACCount™ for CD4 lymphocyte enumeration had the lowest SDI variance and tightest %CV between laboratories. DP PLG/CD4 was shown to have the lowest variance and outlier rate of 2% overall. The Mean SDI noted for PLG/CD4 suggests that reads consistently slightly higher than the pooled reported CD4 results. This finding is in keeping with the direct comparisons noted above where PLG/CD4 read slightly higher than predicate testing (see Tables 5 and 6). As noted in the FACSCount comparison performed here (see Table 8), similar slightly higher Mean-SDI was noted in the FACSCount group indicating that FACSCount reads similar to PLG/CD4, and overall higher than "predicate" CD4 testing. Once again, direct comparison of data presented here supports the findings of the CD4 REQAS analysis where automated algorithm "predicate" methods are shown overall to read lower than the pool. As expected the widest variance and highest outlier rate was noted between laboratories performing conventional DP testing that relies on use of the variable Total Lymphocyte Count (17, 18). As reported previously (19), was the finding that laboratories that use modified "predicate" i.e. CD3/4/8 without CD45 gating, read higher than the pooled CD4 reporting show wide variance of reporting noted similar to conventional lymphocyte based DP testing. A similar analysis was performed for CD4%L between laboratories participating on the CD4REQAS. In the CD4REQAS analysis across the same 8 Trials, as expected, the reproducibility of reporting of CD4%L between PLG/CD4 and "predicate" reporting was similar (data not shown), which confirms findings noted in the direct comparative studies performed here (see Table 3). However 100% outlier rates were noted in group who used algorithm based CD3/4/8 and FACSCount users who falsely reported CD4% of T-cells (CD4 of CD3) instead of CD4%L (data not shown). Improvement over subsequent

trials through follow-up and education to participating sites has resulted in marked improvement of performance in this regard.

CONCLUSIONS

DP PLG/CD4 was previously shown to demonstrated to have good method agreement to both Volumetric and SP "Predicate/ state of the art" CD4 (1). Although PLG/CD4 has been introduced in SP format (2, 3), there were no studies to show the cross platform equivalence of dual to single platform Pan*Leuco*gating for CD4 enumeration. In this report we demonstrate excellent cross platform equivalency between DP and SP PLG/CD4 absolute CD4 counts. A mean Bland Altman (BA) positive bias of 4-5 cells/ul was noted in the range of <500 cells/ul (N=481). This minimal mean bias of 4 cells/ul appears to be independent of platform or instrument (data not shown). Irrespective of CD4 count, across the entire data range of 1- 1831 CD4 cells/ul, DP PLG/CD4 read an average ~1±4% higher than SP PLG/CD4 (MPS=98.9±4.4%, N=811). These very minor differences in cell count are not likely to be clinically significant and for the purposes of the NHLS ARV programme were unlikely to alter therapy related decision-making, especially at the 200 cells/ul level. To confirm this finding, we performed a separate BA analysis in the range of 150-250 CD4 cells/ul (N=112) revealing an actual bias of ~6-7 cells/ul difference. This information facilitated the transfer from DP to SP PLG/CD4 as the predicate methodology within the South African NHLS, whilst still allowing the reference laboratory to continue to perform both systems (important to ensure method/ platform continuity as this centre also functions as a core facility for many research studies in the region). In the PLG/CD4 training workshops that have been conducted and run by our group, we have thus encouraged laboratory personnel using PLG/CD4 to use DP PLG/CD4 as an internal quality control reference, plotted on an ongoing basis in a Levey-Jennings fashion on random SP PLG/CD4 results, plotted at regular intervals, at least on a daily basis.

In the Predicate cross platform analysis, DP Predicate read higher than SP Predicate, as was noted in the similar PLG/CD4 platform comparison confirming that DP overall reads higher than SP, irrespective of gating strategy. However the bias between Predicate was more than in the comparative PLG/CD4 comparison, reading overall 6±5% and ~28 CD4 cells/ul higher than conventional SP "Predicate" in the range < than 500 CD4 cells/ul. Close method comparison noted in the DP vs. SP PLG/CD4 comparison and much tighter confidence intervals noted (attributable to use of total leucocytes as the primary gating step with PLG/CD4 as opposed to use of Total lymphocyte count used in conventional lymphocyte based DP testing], suggests that use of PLG/CD4 gating can reduce counting bias between platforms. Further it suggests that if PLG/CD4 gating is used, that platform may be used interchangeably with minimal bias but that this may not possible with Predicate testing.

Evidence of improved inter-laboratory performance and reproducibility with potential use of PLG/CD4 was noted in the performance of 3 geographically separate US laboratories (Figure 6), where both SP and DP PLG/CD4 as well as SP and DP Predicate testing was performed. The comparison of SP to DP PLG/CD4 in all

three sites showed close method agreement and minimal variation between platforms (%CV range 2.3 to 3.3, Figure 6, No.3). Although in both comparisons of SP PLG/CD4 vs. SP Predicate CD4 and DP PLG/CD4 vs. SP Predicate CD4, good method agreement was noted at the first two sites, noticeable intra-laboratory variation between methods was noted at the third site. This variation was unlikely to be due to pipetting error (no evidence of pipetting error was noted in the SP vs. DP PLG/CD4 analysis demonstrating that the site personnel had good pipetting technique) but suggests variation may have been introduced by gating strategy. This finding is relevant in the context of less or inexperienced laboratories especially in a resource poor context, that are unfamiliar with flow cytometric gating requirements, the subtle complexities of sequential gating strategy or Boolean logic. It further reflects that fewer steps in a particular gating strategy can certainly improve reproducibility of CD4 reporting, as evidenced by the small bias' and tight variance noted with PLG/CD4 usage. Certainly, in the South African context, the simple gating strategy used in PLG/CD4 has enabled implementation into more than 25 sites with the vast majority of sites having no flow cytometry experience. Inter-laboratory reproducibility as evidenced by the good inter-laboratory %CV's of consistently 10-11% or less noted between 25 or more NHLS PLG/CD4 laboratories on the WHO CD4 REQAS programme is testament to the excellent potential for reliable inter-laboratory reproducibility of PLG/CD4, even in "unskilled" hands.

It is important to show that PLG/CD4 has overall similar accuracy to when compared to "Predicate" CD4 systems in order to encourage use of a new gating strategy in sites accustomed to gating used for 20 years or more (20). Good method agreement and a highly significant correlation of more than 98% was noted between the "Predicate"CD4 system and PLG/CD4, irrespective of platform used. On average, PLG/CD4 read between 3-5% higher than "Predicate" CD4 i.e. on average, there was a negative bias of -18 to -23±30 cells/ul using PLG/CD4 in CD4 range less than 500/ul. This reduced to -11 to -13±21 in the 150-250 CD4 cells/ul range. The influence of one platform to another viz. DP to SP, was noted in the PLG/CD4 to PLG/CD4 and in the ""Predicate to "Predicate" comparisons, with instrument variation appearing to play a minimal role (the predominant system used through all sub-studies was a single type of Flow Cytometer viz. BC XL-MCL). Such differences, although minor, still need to be taken into account if a laboratory considers changing over from Predicate CD4 testing to PLG/CD4, in either DP or SP format. These differences should not however be clinically relevant at the therapeutic clinical decision point of 200 CD4 cells/ul, where an expected range of variation of count would be ~11 to 13 CD4 cells/ul between systems based on the studies performed here.

Overall these findings presented here are not very different from the mean negative bias reported previously by our group (-8.4 cells/ul) of the comparison between DP PLG and the full panel (Lymphosum) Volumetric testing, performed on an Ortho Cytoron SP instrument (1) i.e. DP PLG/CD4 was shown to read on average 8 cells/ul higher than volumetric based CD4 testing. The findings reported here however, demonstrate improved accuracy and precision than those reported in a similar study (15) where DP "Predicate" vs. DP PLG/CD4 (N=132) revealed a negative bias of PLG of -56 CD4 cells/ul, and a MPS 109.06%.

In contrast to the Predicate comparison, a 100% similarity was noted in the comparison of both DP and SP PLG/CD4 to the dedicated CD4 counting system, FACSCount™ (N=73). This is not surprising considering that the simple 2-step primary CD3 gating to identify CD4 lymphocytes used on the FACSCount is very similar in concept to the simple, 2-step strategy of PLG (i.e. total CD45, and CD4/SS expression, irrespective of differences of sample preparation (14). Bland Altman analysis revealed only a very small similar bias of +7-cell/ul bias between the technologies in the clinical significant range < than 300 CD4 cells/ul. By comparison, FACSCount™ versus "Predicate" CD4 (BC TetraONE) on the same data set however, showed that FACSCount™ reads higher on average than the state of the art CD4 systems with an overall bias of ~44 CD4 cells/ul (FACSCount used as the standard). The variation around the bias (SD) was however similar for both the state of the art and PLG/CD4 when compared to FACSCount suggesting that some variation of the flow cytometric systems can be attributed to manual gating in both the state of the art and PLG systems, as opposed to the very tight software algorithm gating that is applied in the FACSCount™ (14). Overall these findings were compared in the CD4 REQAS multi-site analysis where FACSCount results were higher on average than laboratories that reported SP Predicate type testing.

In the CD4%L comparison, CD4%L values read on average 1% lower when generated within the PLG protocol as opposed to the CD4%L generated within the "Predicate" system. This finding that the systems are close is not surprising as the PLG strategy uses the same CD45*bright* gate as is used in the Predicate, typically drawn around CD45*bright*/ low SS cells used to identify the CD45 expressing lymphocytes. The difference between the systems however lies in the use of CD3 expression in the "Predicate" system to define T cells vs. use of differential CD4 expression with SS to exclude monocytes in PLG/CD4 (Figure 1). A criticism of PLG/CD4 has been the lack of use of CD3, which can potentially cause PLG/CD4 results to be higher due to false inclusion of CD4 expressing monocytes in the PLG/CD4 cell count. However, the results reported here indicate that is unlikely. Firstly, there was almost no difference noted between "Predicate" vs. PLG with regard to generation of a CD4%L. The CD4%L analysis shows minimal differences between systems, revealing an average bias of less than 1% overall between PLG derived and state of the art derived CD4%L. This finding suggests that CD3 expression may not be necessary to define CD4 lymphocytes and that use of SS in this context is adequate to separate low CD4+/ high SS expressing monocytes from brightly expressing/ low SS CD4 lymphocytes. It further suggests that a CD4%L can be derived without incurring costs or potential variability that could be introduced with additional gating steps related to inclusion of CD3 (as was noted as a potential reason for Predicate intra-method variation noted in Figure 5). Secondly, although PLG was shown to read higher than "Predicate" in both SP and DP comparisons, the influence of platform to platform is likely to be the reason in this context, as evidenced by the similar high bias noted in the equivalent "Predicate" comparison (Table 5).

Use of FCR with SP PLG/CD4 to monitor and exclude possible pipette error has had a significant impact in further ensuring that sites report consistently accurate CD4 results in this context. The lack of algorithm driven

software and "manual" gating of PLG/CD4 has been locally criticized. In contrast, "experienced" sites using more sophisticated algorithm gating and Flow Cytometry systems have performed less well in terms of inter-laboratory reproducibility on the WHO CD4 REQAS when compared to the performance of the PLG/CD4 and laboratories that use dedicated CD4 systems (Table 9). It seems logical where Flow Cytometry skills are lacking that algorithm driven systems would appear to be a good option for standardizing flow cytometric CD4 systems. However, the findings of the CD4 REQAS programme presented here reveal that laboratories using algorithm based detection systems in the hands of local South African and African users, perform less well on the EQAS programme, in contrast with the potential performance of the technology in experienced hands (13, 16). This can be attributable locally to due to lack of insight of use of the algorithm, lack of ability to manually override the algorithm and system if required or poor knowledge of specific gating, all of which can equally negatively influence the performance of the laboratory. Therefore simple gating, taught with insights into trouble shooting is valuable in ensuring transfer of skills necessary to run a PLG/CD4 service. Encouraging was that the %CV of DP PLG and SP PLG noted (9.8 & 10.7% respectively) was only slightly higher than the very reproducible dedicated CD4 instrument, FACSCount (13), showing a %CV =7.6%. This instrument, like PLG, uses a simple 2-step gating strategy, including built-in quality control in the form of duplicate bead readings to ensure accurate pipetting (13), similar in concept to use of FCR in SP PLG/CD4. Both systems had low outlier rates. The reliable reproducibility noted in the PLG/CD4 user group further highlights that "manual" flow cytometric systems (pipetting and gating) can be utilized in laboratories at district hospital level, even where there is little or no flow cytometry background of personnel. Systems like PLG/CD4 that utilize simple gating and manual pipetting can therefore be implemented widely, on a large scale, to accommodate high volumes required to support ARV treatment programmes at a national level in countries with limited resources like South Africa. The PLG/CD4 system is easily implemented and taught, and can be highly reproducible between laboratories (as has been done within the National Health Laboratory Services to support the National Department of Health ARV treatment programme in South Africa).

In summary, we have shown that PLG/CD4 compares well, showing excellent reproducibility between laboratories, irrespective of platforms and gating strategy used i.e. between "Predicate" CD4 methods. PLG/CD4 gating has numerous advantages. Cost reduction potentially has the most impact, especially in a resource-poor/ cost-constrained setting. Further, the simplicity of the gating strategy has a dual role to play: potentially it can improve both intra- and inter-laboratory reproducibility, and certainly within the context of DP testing, can allow for continued use of DP with matched inter-laboratory reproducibility of SP systems (2, 3, 4) in laboratories that prefer to use DP format. The simple PLG/CD4 gating strategy is easy to teach, even in a setting where skills are severely limited. The ease of implementation of PLG/CD4 noted in the South African setting is testament to this. In this context, the use of PLG/CD4 can ensure a sustainable, reliable, robust and cost effective CD4 enumeration service to guarantee the delivery of an effective and safe ARV treatment programme in South Africa.

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32.

FIGURES

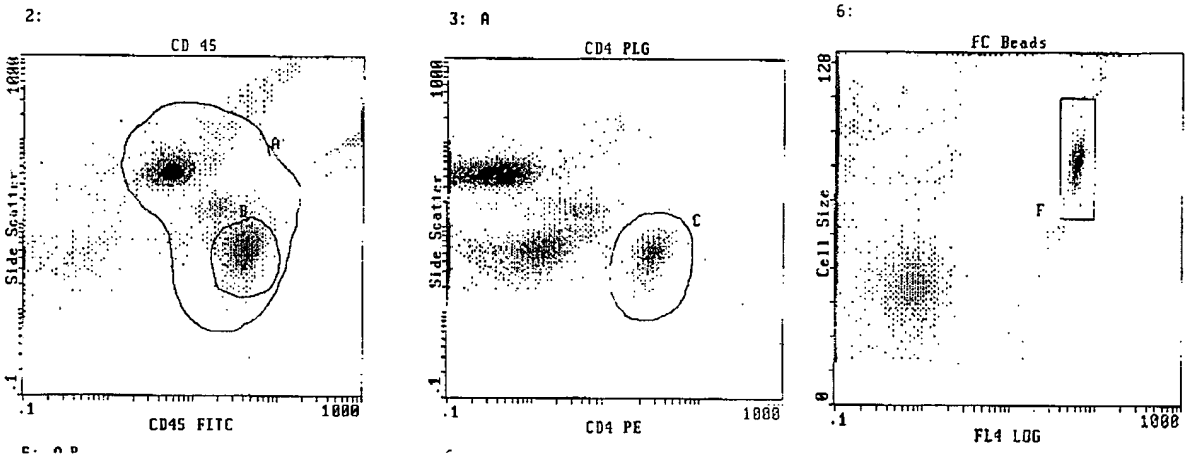


Figure 1. PLG/CD4 gating strategy.

Total leucocytes are initially identified on a [CD45 *vs.* side scatter (SS)] plot (Region A, Histogram "CD45"). All gated events in Region A are then displayed in the next Histogram "CD4 PLG" using a [CD4 *vs.* SS] display where CD4++ lymphoid cells are identified (Region C, CD45). Monocytes are excluded by virtue of their dim CD4 expression and higher side scattering properties. In this method, all white blood cells serve as the reference/ 'common denominator' for DP. For DP PLG/CD4 absolute counts, the CD4% of total leucocytes (CD4%PLG) viz. Region [B] divided by Region [A] is multiplied by the WCC obtained from a haematology analyser to derive an absolute CD4 count.

As shown in this figure, SP can also be applied using the same gating strategy. Beads are identified in the Histogram labeled "FC Beads", Region "F"

PLG/CD4 differs from state of the art in that the reference population used is total white blood cells (Gate A) as opposed to the state of the art method that uses the bright CD45*bright* expressing lymphoid population (Gate B) as the point of reference.

The PLG/CD4 gating strategy also provides for the calculation of a CD4% of lymphocytes, i.e. the number of events in Region C (Histogram "CD4 PLG") divided by numbers of events in Region B (Histogram "CD45"). This value is however not used in the calculation/ generation of the PLG/CD4 counts but is derived as additional information when CD4% of lymphocytes may be clinically relevant, e.g. in pediatric samples.

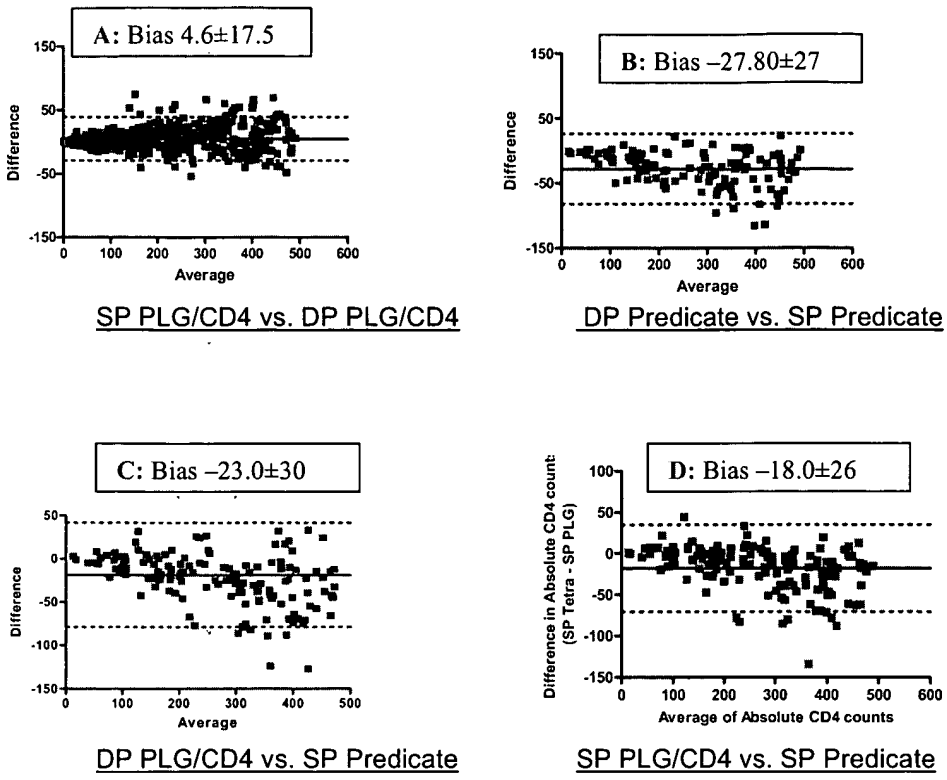


Figure 2: Bland Altman analysis in clinically significant range < than 500 CD4 cells/ul comparing

[A] SP PLG/CD4 versus DP PLG/CD4 (N=481),

[B], DP Predicate vs. SP Predicate, (N=111).

[C], DP PLG/CD4 vs. SP Predicate, (N=142) and

[D], SP PLG/CD4 vs. SP Predicate, (N=145),

See Tables 4-7 for details of statistical analysis.

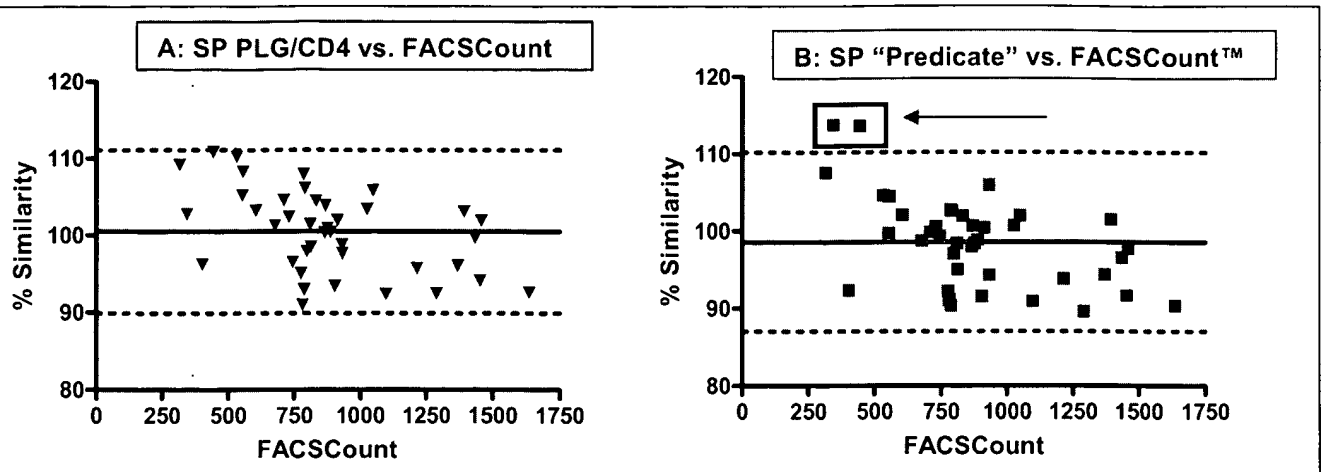


Figure 3: Percentage Similarity Model analysis comparing SP PLG/CD4 [A] or SP "Predicate" [B]) versus FACSCount™CD4, as the standard. There were no outliers in the SP PLG/CD4 versus FACSCount™ analysis (MPS 100.7±5.3; %CV=5.3). Two outliers (highlighted with an arrow) are noted on the FACSCount versus Predicate comparison (MPS 99.0±6.0; %CV=6.1).

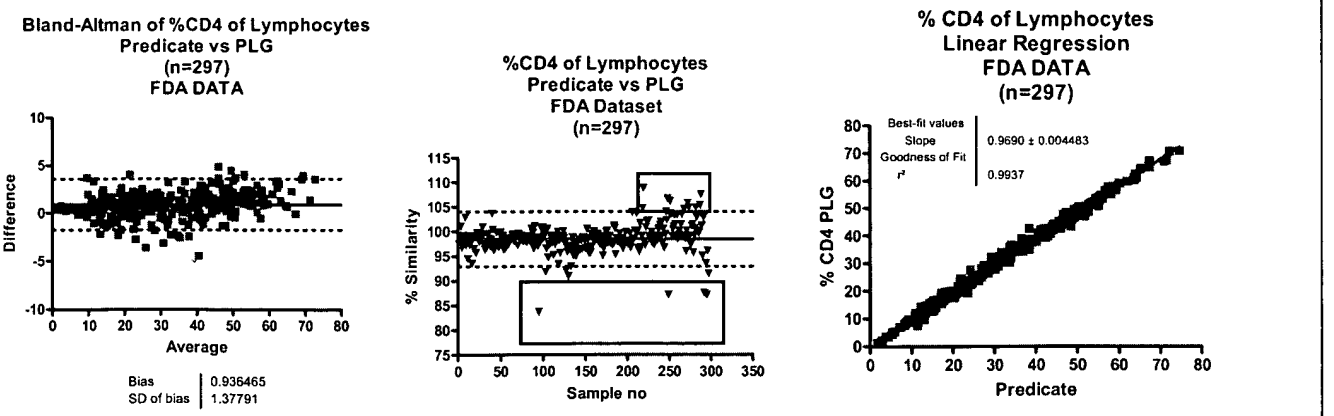


Figure 4: Statistical analysis comparing CD4%L in PLG/CD4 vs. "Predicate" CD4%L as well as Predicate vs. Predicate CD4%L (figure still needs fixing in Adobe Illustrator and tidying up). Suggestions welcome

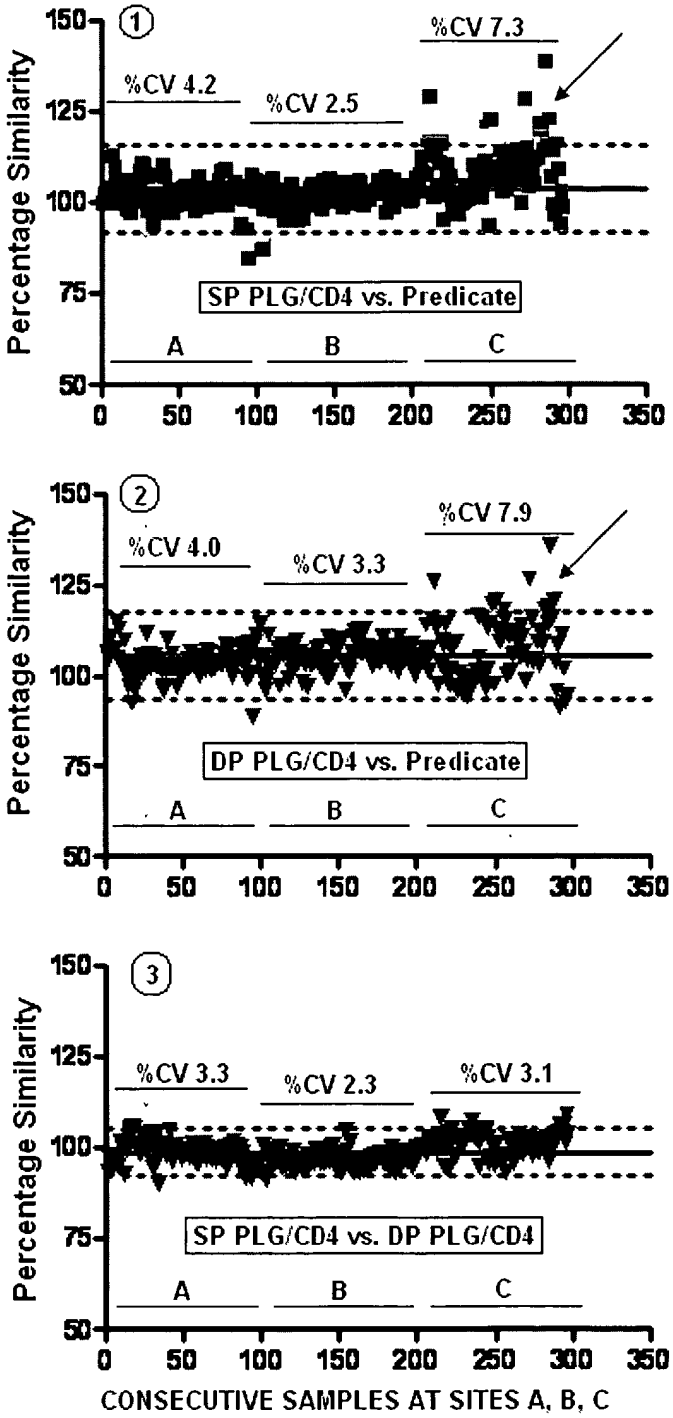


Figure 5: Figure of Percentage Similarity/ MPS (data set C) versus consecutive samples prepared and analysed in 3 separate geographic sites: A, B, and C. There is close method agreement and less variation between platforms using PLG/CD4, (No.3) with tighter %CV of percentage similarity noted. Of note is the higher %CV's noted at site C (Nos. 2, 3), in the predicate comparisons, which is not present in the SP PLG/CD4 vs. DP PLG/CD4 analysis and not due to pipette error, and suggesting variation introduced by "Predicate" gating. This variation is unlikely to be due to pipetting error, as there is no outliers or evidence of pipetting error noted in the SP PLG/CD4 vs. DP PLG analysis, where SP and DP values compare well (implying the site has adequate pipetting technique). Additional gating steps may contribute to variability, especially where manual gating is performed and in this context, predicate testing could potentially result in more variable results, especially in inexperienced laboratories. Use of the TLC used in DP "Predicate" calculation may also be contributing to variation noted in this site. This finding is in contrast to the tight %CV noted within the PLG analysis reflecting use of WCC and perhaps reflecting that fewer steps in a particular gating strategy can certainly improve reproducibility of CD4 reporting.

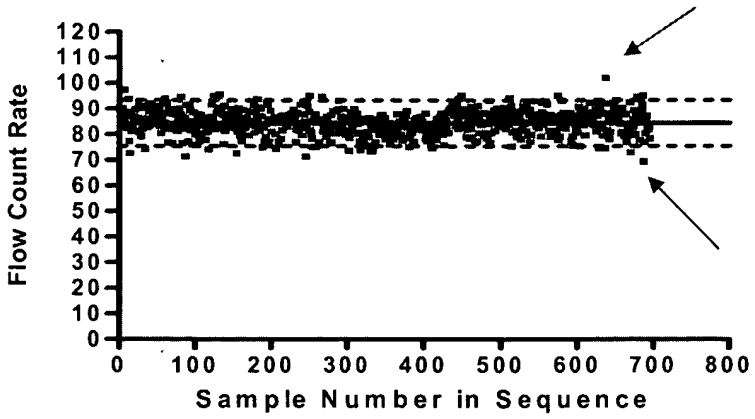


Figure 6: Flow Count Rate/ FCR obtained by dividing the total number of beads counted by the time of the flow cytometric analysis. These results are plotted in sequence of preparation and analysis of 694 consecutive samples analysed (separate study viz. data used, with permission, from the Johannesburg Health Care workers Study, March 2005). Remarkable consistency of FCR/bead rate is demonstrated over time showing stability and accuracy of pipetting technique at the Johannesburg site; a mean FCR of 84.4 beads/ second is noted, with a Standard Deviation (SD) of 4.6 and %CV of 5.5%.

A FCR result of a sample that falls more than 2 SD outside of the Mean of the previous 100 samples, indicates possible pipette error, and should be re-prepared and re-analysed. Such outliers are easily identified to ensure the quality control of the manual bead pipetting step Practically, the FCR data can be transferred through an electronic data interface, to the laboratory management system for ongoing automatic quality control monitoring, both at the individual sample level in the laboratory where the test was performed to ensure no pitting error occurred, as well as facilitating ongoing site monitoring from a centralised Quality Assurance division. Red arrows in this figure indicate possible outliers for attention prior to CD4 result release.

TABLES

Table 1: Details of the study sets analysed in Johannesburg and in the US (Miami and FDA data), including sample numbers, mean (median) CD4 cell counts, *range of CD4* counts, instruments and reagents used.

Individual Data SET		Evaluation of Cross Platform equivalency DP PLG/CD4 *vs.* SP PLG/CD4		Evaluation and Comparison of Accuracy: DP PLG/CD4 *vs.* #SP Predicate CD4		Evaluation and Comparison of Accuracy: SP PLG/CD4 *vs.* #SP Predicate CD4		CD4 counts (Predicate) Mean (median) in Cells/ µL, *CD4 range*
	STUDY Location	**N**	**Instrumentation reagents**	**N**	**Instrumentation reagents**	**N**	**Instrumentation reagents**	
A	[1]BCI, Miami	**46**	BC XL MCL	**46**	TetraONE™ XL MCL	**46**	TetraONE, XL MCL	625 (613) *82-1393*
B	[1]BCI, Miami	**46**	FC500	**46**	TetraONE™ CXP FC500	**46**	TetraONE CXP, FC500	644 (638) *84-1407*
C	[2]US FDA 510(k) BCI,	**297**	BC XL MCL	**297**	TetraONE™ BC XL MCL	**297**	TetraONE BC XL MCL	617 (603) *15-1850*
D	[3]NHLS Johannesburg	**322**	BC XL MCL	-	-	-	-	256 (178) *1-1605*
E	NHLS Johannesburg	**100**	BC XL MCL	-	-	-	-	294 (223) *2-1831*
F	NHLS Johannesburg	-	-	**33**[3]	BDIS FACSCount™	-	-	336 (225) *3-1214*
H	NHLS Johannesburg	-	-	-	-	**40**[3]	BDIS FACSCount™ & TetraONE BC XL MCL	825 (799) *219-1637* 787 (752) *195-1436*
	TOTAL N	**811**		**389** (416)		**389** (429)		

Footnotes:

- SP Single Platform, DP = Dual Platform testing utilizing a BCI GenS Haematology analyser
- All XL and FC500 Flow Cytometry, TetraONE and FlowCARE PLG and Immunoprep are registered products or Flow Cytometers of Beckman Coulter International (BCI), Miami, FL, US. All SP PLG/CD4 or SP "Predicate" (TetraONE) performed on BC XL use FlowCOUNT™ beads, (BCI, Miami, FL).

[1]Miami (20 HIV+ and 26 normals)

[2]US FDA 510(k), performed in 3 separate geographical sites comparing SP CD45/3/4/8 (TetraONE, BCI, Miami, FL) with DP and SP PLG/CD4, using XL MCL with for both PLG and Predicate CD4 lymphocyte enumeration. PLG/CD4 performed using FlowCARE PLG/CD4 CD45 - BC3821F4A-FITC and CD4 SCF|12T4D11-PE with ImmunoPrep, (BCI, Miami, FL, US), comprising XX HIV+ and XX

[3]Comparison to FACSCount, also performed as a separate analysis

Table 2: FlowCOUNT ™ Bead reproducibility (11) of a fresh, normal sample prepared 10 times. All replicates were analysed in order of preparation on a XL MCL Flow Cytometer (BCI, Miami, FL). Based on the experience of the Johannesburg laboratory a %CV of bead rate < than 5-6% is acceptable to ensure accuracy of count and exclude pipette error in a routine context (11).

No.	Time In secs	Bead count	CD45/ µL	ALC/ µL	Absolute CD4/ µL	FCR/ Bead rate
1	42	3919	4974	1277	574	93.31
2	42	3625	5157	1381	603	86.31
3	42	3840	4947	1303	575	91.43
4	42	3799	4971	1317	598	90.45
5	41	3545	5236	1412	627	86.46
6	42	3873	4846	1292	582	92.21
7	42	3790	5041	1321	604	90.24
8	42	3774	5046	1326	595	89.86
9	43	3893	4868	1286	580	90.53
10	43	3902	4880	1283	565	90.74
Mean	**42.1**	**3782.3**	**4999.1**	**1324.6**	**592.1**	**89.8**
SD	**0.6**	**122.3**	**133.8**	**44.2**	**18.7**	**2.1**
%CV	**1.4**	**3.2**	**2.7**	**3.3**	**3.2**	**2.3**

#Stop typically set at 5000 lymphocytes (CD45*bright* gated).

Table 3: Comparison of CD4% of lymphocytes (CD4%L). Use of CD45*bright* with CD3+/CD4+ gating used in "Predicate" is compared to alternative use of CD4 expression and Side Scatter (SS) in the PLG/CD4 protocol (CD4/SS within bright CD45 gated cells) to identify CD4%L. Both protocols utilise bright CD45 gating as the first step to immunophenotypically define lymphocytes. Correlation and Linear Regression, Bland Altman and Percentage Similarity Statistics are presented.

CD4%L Comparison	**N**	**MPS**	**%CV MPS**	**Bland Altman analysis CD4%L (*LOA)**	**Correlation r [Linear Regression] (R^2)**
PLG/CD4 (XL) vs. [1]Predicate (XL) (No outliers excluded – see Table 3	[2]389	98.6±2.71	2.75	0.9±1.43%	0.996* [y=0.974x+0.00] (0.993)
[3]Predicate XL vs. [4]Predicate FC500	[3,4]46	101.2±2.07	2.05	-0.9±1.71%	0.996* [y=1.05x+0.01] (0.992)
[3]Predicate XL vs. [4]Predicate XL	[5]297	100.1±1.74	1.74	0.03±0.89%	0.999* [y=0.998x+0.00] (0.997)

[1]Predicate = CD4% of bright CD45 gated, CD3 gated population; [2]Comprised of data sets A, B and C (Table 1) [3,4]Comparison of data sets A and B (Table 1). [5]Comparison of Predicate CD4% replicates - data set C (Table 1). *LOA (95% Limits of Agreement). All analyses were performed on a BC XL-MCL flow cytometer (XL).

Table 4: Table of comparison of SP PLG/CD4 versus **DP** PLG/CD4[#]

	N	MPS±SD	%CV MPS	Bland Altman (LOA) CD4 Cells/μL	Correlation r [Linear Regression] (R^2)
All data including data sets A, B, C, D and E (Table 1)	811	98.9±4.4%	4.42%	9.6±48.2 (-84.9 to 103.8)	0.992* [y= 0.987x + 0.004] (0.985)
Clinically significant CD4 range **less than 500 cells/ul**	481	99.0±4.7%	4.8%	4.6±17.5 (-30 to 39)	0.992 * [y= 0.975x + 0.005] (0.984)
Clinically significant CD4 range **150-250 cells/ul**	112	98.6±3.9%	4.0%	6.7±16.7 (-26 to 40)	ND

[#] Previously published DP PLG/CD4 was used as the standard for comparison (1); * p<0.001, noted to be a highly significant correlation; *LOA (95% Limits of Agreement)

Table 5: Table of comparison of DP "Predicate" versus SP "Predicate" CD4[#]

	N	MPS±SD	%CV MPS	Bland Altman (LOA) CD4 Cells/μL	Correlation r [Linear Regression] (R^2)
All data including data sets A, B, C (Table 1)	297	104.6±5.9%	5.7%	-41±67.7 (-174 to 91)	0.981* [y =0.989x + 0.01] (0.965)
Clinically significant CD4 range **less than 500 cells/ul**	111	106.0±5.7%	5.3%	-27.8±27.0 (-82 to 27)	0.982* [y =1.055x + 0.02] (0.962)
Clinically significant CD4 range **150-250 cells/ul**	22	107.0±5.1%	5.7%	-24.9±40.7 (-66 to 16)	ND

[#] SP Predicate used as the standard for comparison; * p<0.001, noted to be a highly significant correlation; *LOA (95% Limits of Agreement)

Table 6: Table of comparison of DP PLG/CD4 versus SP "Predicate" CD4[#]

	N	MPS±SD	%CV MPS	Bland Altman (LOA) CD4 Cells/µL	Correlation r [Linear Regression] (R^2)
All data including data sets A, B, C (Table 1)	389	105.3±5.6%	5.4%	-63±78.0 (-216 to 89)	0.981* [y=1.059 + 0.01] (0.963)
Clinically significant CD4 range **less than 500 cells/ul**	142	104.5±5.7%	5.5%	-23±30.0 (-83 to 35)	0.975* [y=1.080 + 0.02] (0.952)
Clinically significant CD4 range **150-250 cells/ul**	44	103.3±5.9%	5.7%	-11±22.9 (-56 to 33)	ND

[#] SP Predicate used as the standard for comparison; * p<0.001, noted to be a highly significant correlation; *LOA (95% Limits of Agreement)

Table 7: Table of comparison of SP PLG/CD4 versus SP "Predicate" CD4[#]

	N	MPS±SD	%CV MPS	Bland Altman (LOA) CD4 Cells/µL	Correlation r [Linear Regression] (R^2)
All data including data sets A, B, C (Table 1)	389	104.5±5.8%	5.6%	-53.79±78.9 (-133 to 25)	0.981* [y =1.068 + 0.01] (0.963)
Clinically significant CD4 range **Less than 500 cells/ul**	145	103.2±5.6%	5.4%	-18±26 (-70 to 34)	0.980* [y =1.068 + 0.01] (0.962)
Clinically significant CD4 range **150-250 cells/ul**	31	103.7±5.9%	5.7%	-13±21 (-56 to 29)	ND

[#] SP Predicate used as the standard for comparison; * p<0.001, noted to be a highly significant correlation; *LOA (95% Limits of Agreement)

Table 8: Table of comparison of SP PLG/CD4, DP PLG/CD4 or SP "Predicate" versus FACSCount™

PLG versus FACSCount Statistical analysis	N	MPS±SD	%CV MPS	Bland Altman (LOA) CD4 Cells/µL
Data Set F: (Table 1) DP PLG/CD4 vs. FACSCount	33	100±7.9	7.9	7.4±44.6[$] (-79 to 94)
Data Set G: (Table 1) SP PLG/CD4 vs. FACSCount	40	100.5±5.3	5.3	6.68±94.0[#] (-177.6 to 191.0)
Data Set G: (Table 1) SP Predicate vs. FACSCount	40	98.6±5.9	6.0	43.9±103.0[#] (-158.1 to 245.8)

For the MPS and BA analysis FACSCount™ is used as standard for comparison. [5]Three outliers were noted, 2 of which were not clinically significant and in a > than 800 CD4 cells/ul range. # A single outlier were not clinically relevant and noted to be in the range > than 1500 CD4 cells/ul. *LOA (95% Limits of Agreement)

Table 9: Table of performance of laboratories performing various CD4 methodologies, cumulative over 8 trials of the WHO CD4 REQAS. PLG/CD4 reproducibility between laboratories is demonstrated in an SDI analysis of performance of laboratories performing the same technology.

Absolute CD4	**Pooled N (Trimmed N)**	**Outlier Rate**	**Trimmed SDI Analysis MEAN±SD**	***Trimmed analysis of absolute CD4 results: %CV**
SP *PLG*/CD4[1]	109 (105)	3.7%	0.1±1.1	**10.7**
DP *PLG*/CD4[1]	51 (50)	2.0%	0.3±0.8	**9.8**
SP- FlowCOUNT™ [2](Exl. PLG[1])	120 (113)	5.8%	-0.1±0.9	**12.6**
All DP (Exl. PLG[1])	77 (69)	10.4%	0.0±2.1	**13.5**
Automated algorithm[3] **SP** Predicate i.e. *CD45/3/4/8*	43 (40)	[5]7.0%	-0.3±2.0	[5]**13.9**
Automated algorithm[3] **SP**, *including CD3/4/8 without CD45*	92 (85)	[6]7.6%	0.4±2.1	[6]**17.1**
Dedicated FACS*Count*™[4]	40 (39)	2.5%	0.2±1.1	**7.9**

Footnotes to Table 9:

[1]FDA approved PLG/CD4 CD4, BCI, Miami, FL. Majority of laboratories utilise BC XL-MCL flow Cytometers. PLG/CD4 requires some operator input on gating; SP = Single Platform; DP = Dual Platform PLG variations reported.

[2]FlowCOUNT™ BCI, Miami, FL, including results submitted from sites using both older international guidelines (CDC/ NCCLS) i.e. lymphocyte based light scatter (LS) gating with CD3/4/8 and laboratories that use current international guideline (CDC) based CD45 gating with CD3/4/8;

[3]*Automated Algorithm driven system* - Trucount™/ MultiSET™ BDIS, San Hose, CA using CD45/3/4/8 or CD3/4/8 *without* CD45 [in the hands of African users];

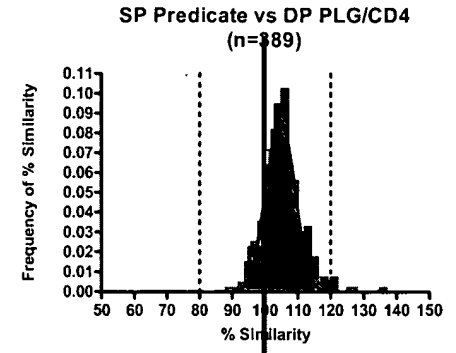
[4] FDA approved FACSCount™, Becton Dickinson Immunocytometry Systems (BDIS), San Hose, and CA;

[5] Higher Outlier Rates and higher %CV of the Trimmed analysis of this group indicate that automated algorithm systems do not appear to be optimally used (in the hands of South African and other African users). Further training on these systems, at these sites, is recommended to ensure users have insight into use of the algorithm, especially in stressed samples, such as those that may be received routinely or on an EQAS program. [6]A higher outlier rate and higher %CV was noted between laboratories that only CD3 (but not CD45) in the algorithm based SP CD4 system.

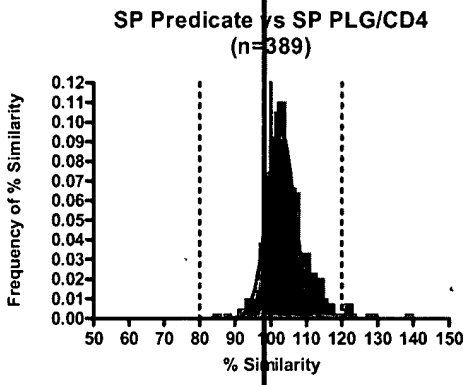
*As a point of reference of performance, the mean Trimmed %CV across all participants and over 8 trials was 12.9%

Lastly,

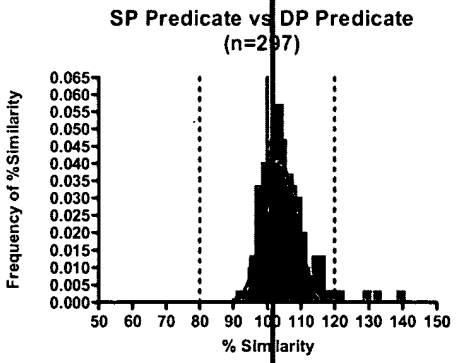
I was thinking of including this figure instead of BA analysis (Figure 2) as I think graphically the Percentage Sim model shows is more visual in showing where data sets compared read high or low; outliers can still be easily seen



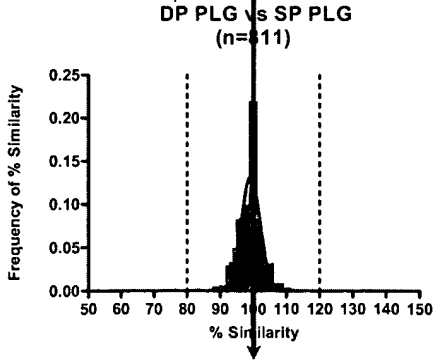
DP PLG/CD4 vs SP Predicate- N=389



SP PLG/CD4 vs SP Predicate- N=389



ADD DP PRED vs. SP Predicate



SP PLG/CD4 vs DP PLG/CD4

100% LINE OF SIMILARITY